SUMMARY

Sunflower (*Helianthus annuus* L.) is the most important oilseed crop in South Africa at present and is grown in all summer rainfall areas. Sunflower seed oil is preferred over soybean and rapeseed oil because of its high quality and high poly-unsaturated fatty acids content that helps avoid the accumulation of cholesterol in the blood (Ward *et al.*, 1985). A field trip was taken to sunflower fields in Greytown, Northern Kwa-Zulu Natal in early March 2010 during the warm and rainy summer season. *Alternaria helianthicola* Rao and Rajagopalan was consistently isolated from diseased plant material. *Alternaria helianthicola* has not previously been recorded on sunflower in South Africa. The pathogenicity of was *A. helianthicola* confirmed on sunflower plants using Koch's postulates. Standard germination and seed health tests were conducted for thirteen sunflower seed lots from various sunflowers farms and companies of South Africa. Germination percentages ranged from 60 to 94% and germinated seedlings of the thirteen seed lots often showed seedling blight. Seed infection ranged from 18 to 98% caused by various small-spored *Alternaria*species. Seed infection did not severely influence seed germination and the *Alternaria* species may either cause a quiescent infection of the seeds or the*Alternaria* species may be mere saprobes and contaminants of the seed coats that do not cause disease. Seed component plating tests showed that the *Alternaria* species were more prevalent in the embryo and cotyledon than on the seed coats. Morphological characterization of these small-spored *Alternaria* species has been found to be unreliable due to the overlap in cultural characteristics between the various species. Molecular characterization using the rDNA ITS operon, β-tubulin gene and the EF-1α gene was done to support the morphological characterization. The rDNA ITS operon showed extensive length polymorphism among the *Alternaria* species that did not allow proper molecular identification of the isolates. The *in vitro* test showed that *A. helianthicola* had an optimum growth temperature of 25°C and maximum temperature of 35°C. Light was observed to promote hyphal growth increasing the radial growth rate of the fungus. In *in vivo* tests, approximately 12 hours of continuous high RH was required for infection to progress at optimal temperatures. Temperature had a significant effect on infection, with lesion development and enlargement observed to increase from 20 to 30°C, declining at 35°C.

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Chapter 1: General introduction

The agriculture sector contributes about 3% of the gross domestic product (GDP) of the South African economy (Hannon and Cassel, 2012). In South Africa, the sunflower (*Helianthus annuus* L.) crop is the third most important field crop after maize (*Zea mays* L.) and wheat (*Triticum aestivum* L.) and is the most important oil crop (Grains South Africa, 2010). For the periods between 2001 and 2011, the local annual production of sunflower seed ranges between 500 000 to 700 000 tons and the quantity of the sunflower oil represents about 82% of all edible oil produced in South Africa while the gross value is approximated at 1.5 billion rands per anum (DAFF, 2012a).

The cultivated sunflower ranks with soybean [*Glycine max* (L.) Merr.], rapeseed (*Brasicca rapa* L.), and peanut (*Arachis hypogaea* L.) among the four most important annual crops in the world grown for edible oil (Carter, 1978). Sunflower is the worlds second most produced edible oil crop. The quantity of the sunflower oil represents about 14% of the total world production of the major vegetable crops (Robbelen *et al.*,1989).

The major production areas in South Africa are North West, Free State, Limpopo and Mpumalanga (DAFF, 2012b). The sunflower crop is propagated by seeds. It is recorded that more than 24 fungal speciesare seed-borne in the crop. One of the predominant seed-borne pathogens is *Alternaria* species. *Alternaria* spp. are the cause of leaf blight of sunflower (Nahar *et al.,* 2005). They are known to infect all parts of the plant viz., leaf, petiole, stem, all flower parts, and seeds (Kim and Mathur, 2006). Sunflower seed infection with *Alternaria* spp. causes biodetorioration and reduction in germination in the seeds (Ojiambo *et al.,* 1998).

Alternaria species are the cause of many plant diseases and yet other *Alternaria* species are said to be saprophytic (Rotem, 1994). The knowledge of the systematics and classification of plant inhabiting fungi is fundamental for making appropriate plant quarantine decisions (Palm, 2001). The early and accurate diagnosis of plant disease is the most crucial component of any crop management system because plant diseases can be managed most effectively if control measures are introduced at an early stage of disease development (Miller and Martin, 1988).

Research in the present study aims at providing information on *Alternaria helianthicola*, the causal agent of Alternaria leaf spot of sunflower and are presented in the following chapters.

Chapter 3: Field trips to sunflower farms in Northern KwaZulu-Natal and Potchefsroom confirmed an extensive leaf spot disease on sunflower leaves. Isolations were made from diseased material collected during the field trips, Koch's postulates were proved and the causal agent was identified as *A. helianthicola* Simmons E.G. The objective of this chapter was to describe the foliar disease and to develop a disease rating scale that could be used to determine the disease severity in a sunflower field.

Chapter 4: Seed quality and seed health are parameters that need to be assessed to determine seed performance in order to assist farmers grow the best crop (ISTA, 2012). Chapter 4 aims to determine the seed health and quality of 13 sunflower seed lots. Morphological techniques identified *Alternaria* spp. to be the cause of seedling blight. These species were small-spored *Alternaria.*

Chapter 5: Aims to use molecular diagnosis of small-spored *Alternaria* species isolated from infected sunflower seeds and leaves to supplement morphological identification.

Chapter 6: The three most important factors in the development of disease are host, pathogen and the environment. Chapter 6 evaluates the environmental conditions that are conducive for disease infection and proliferation of *A. helianthicola*.

Chapter 7: The final chapter is the general discussion which concludes the investigations and studies done on *Alternaria helianthicola*, with recommendations and implications for industry and further research.

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Chapter 2: Literature review

2.1. The Host: *Helianthus annuus* L.

2.1.1. Introduction

Sunflower, the most important oilseed crop in South Africa at present, is grown in all summer rainfall areas (Pannar Seed, 2012). Sunflower derives most of its economic value from the oil extracted from the seed. The oil is considered of very high quality and generally sells for a premium in world markets over soybean and rapeseed oils (Robbelen *et al.*,1989). The production forecast for sunflower seed in South Africa is up to 7 00 000 tons per annum (DAFF, 2012). Sunflower is produced in Limpopo, North West, Mpumalanga and Free State (Esterhuizen and Sindelar, 2010).

2.1.2. Nomenclature, origin and distribution

The sunflower is a member of the Asteraceae (formerly known as Compositae), a large family of flowering plants occurring throughout the world, although a few are of economic importance (Weiss, 1983). The genus *Helianthus* which is named from the Greek *Helios* meaning sun, and *anthos* flower, originated in the south–west United States-Mexico area (Weiss, 1983; Robbelen *et al.*, 1989). The sunflowers of the genus *Helianthus* have 67 species all of which are native to North and South America and 17 of which are cultivated (Weiss, 1983). Two of these species, *H. annuus* L., the common sunflower, and *H. tuberosus* L., the Jerusalem artichoke, are cultivated as food plants and several species are grown as ornamentals (Carter, 1978).

The sunflower was introduced to Europe in 1568 (Heiser, 1955). Sunflower was developed as an important source of oil during the early 1800s in Russia. The Soviet breeders improved the oil content of the seeds from less than 30% to over 50% which lead to the sunflower's development as an important oilseed crop all over the world (Robbelen *et al.*, 1989). Today, the sunflower is produced in many parts of the world including South Africa. South Africa is ranked number nine in sunflower production after Ukraine, the European Union, Russia, Argentina, Turkey, China, Pakistan and the United States (http://www.indexmundi.com/agriculture). South Africa is not a significant role player in the production and trade of oilseeds in the international market as it

contributes only about 3% to the sunflower seeds produced in the world (Grain South Africa, 2010).

2.1.3. Botanical description

The cultivated sunflower is a tall erect, unbranched, coarse annual, with a distinctive large, golden head. The root system of a mature plant is substantial but shallow, and the taproot which develops from the radical of the seed can be up to 3 m in length although it rapidly reduces in diameter from the soil surface (Carter, 1978; Weiss, 1983). The stem is robust and circular reaching up to 10 cm in diameter, bearing rough hairs, and may have slight longitudinal ridges. The stem has a woody exterior that is filled with stiff white pith and frequently becomes hollow with age. The stem is green and generally grows up to 3 m in height (Weiss, 1983).

The leaves are large, ovate, occasionally opposite on the lower stem and alternate above and are carried by long petioles. The leaves are green and some 20 to 40 leaves may be produced per plant. Leaves vary in size, shape of entire leaf, shape of leaf tip and base, shape of margin, and hairiness (Carter, 1978). Leaf production and stem elongation continue until the inflorescence opens and flowering begins (Weiss, 1983; Ramanathan, 2004)

The inflorescence is a capitulum or head, characteristic of the Asteraceae family. The sunflower head is found on the terminal end of the stem and has a large inflorescence. The inflorescence of the sunflower consists of an outer whorl (Weiss, 1983; Robbelen *et al.*, 1989) and the head size varies between cultivars, seasons and soil types, etc. Head diameter has the most significant effect on seed yield, but there is usually optimum diameter for maximum seed production in the field. The head consists of 300 to 1000 flowers but can be higher in non-oil cultivars (Ramanathan, 2004)

The achene or fruit of the sunflower consists of a seed, often called the kernel, and adhering pericarp, usually called the hull (Carter, 1978). The seed consists of a seed coat, endosperm and embryo. The seed coat consists of a spongy parenchyma covered by inner and outer parenchymal layers. The endosperm consists of a layer of aleurine cells coalesced with the seed coat. The embryo is made up of cotyledons (Carter, 1978).

2.1.4. Cultivation conditions

The yield of sunflower is determined by heredity and environment. Heredity is more controllable than environment because it is fixed when the cultivar is chosen, whereas the environment is partially controllable (Carter, 1978). Sunflower will grow on a wide range of soil types provided they are well drained or not acidic (Ward *et al.*, 1985). The sunflower cultivation has been limited to soils where the clay percentage varies between 15 and 55%, thus, in sandy loam to clay soil types. At present the major planting areas are in soils with a clay percentage of less than 20% (Ward *et al.*, 1985). Sunflowers require adequate light. Sunflowers that have been grown in shade that limits 40% of the natural light suffered a 64% reduction in yield (Carter, 1978).

Water requirement varies among years and locations because transpiration rate is affected by the aerial environment-humidity, temperature, wind and light. It is a measure of the crop's efficiency in using water when soil moisture is at optimum level (Carter, 1978). Sunflower is not drought tolerant but often produces satisfactorily even when other crops are seriously damaged. This is due to the crop's extensively branched taproot system (Carter, 1978). However, the oil yield is reduced if plants are allowed to be stressed during the main growing season and at flowering. This causes a reduction in the size and number of leaves by shedding the lower leaves (Weiss, 1983).

Sunflower performs well in temperate zones and it is able to tolerate both cold and high temperatures. Sunflower grows well within a temperature range of 20 to 25°C, although a range of 8 to 34°C is tolerated without significant yield reduction. Emergence is faster at 15°C than at 10°C (Carter, 1978). High temperatures (40 or 45°C) have a deleterious effect on germination and seedling growth perhaps because most enzymes are inactive in such high temperatures. Achenes can germinate at 40°C, but root and hypocotyl growth is strongly inhibited even when the seedlings are later transferred to 25°C (Robbelen *et al.*, 1989).

2.1.5. Harvesting

The most important economic characteristics of sunflower are days to maturity, seed yield and oil content (Ramanathan, 2004). Sunflower is usually harvested 90 to 160 days after planting, depending on the variety (Weiss, 1983). Evenness of growth, ripening and moderate height are

essential requirements for successful mechanical harvesting (Weiss, 1983). At maturity, the heaviest achenes with the highest oil content are in the outer circular positions and the centre of the head usually has unfilled achenes (Murphy, 1994). Sunflowers are harvested after they begin to desiccate (Ward *et al.*, 1985).

2.1.6. Uses of the sunflower crop

The oil extracted from the seed contributes to 80% of the total value of the crop (Murphy, 1994). The oil in sunflower is high in poly-unsaturated fatty acids and is reported to be an important dietary requirement to avoid the accumulation of cholesterol in the blood (Ward *et al.*, 1985).The oil content of sunflower seed is between 25 and 48%, and the protein content is between 15 and 20%, therefore 28g (one ounce) of sunflower seeds contains 6 g of protein, 14g of fats, 2.4g of fibre and 5g of carbohydrates (Weiss, 1983).

Sunflower may be used as food stuffs such as oil and margarine or in snacks and baking as toppings or animal feeds as a source of roughage. Sunflower has also been used industrially for the production of biodiesel; for fuel to generate steam or electricity; in the production of ethyl alcohol; applied in paints and varnishes and used in soaps and cosmetics (Robbelen *et al.*, 1989).

2.1.7. Sunflower diseases

Sunflower is the known host of more than 35 infectious microorganisms, mostly fungi, which may impair the normal physiology of the plant so that the yield and quality are reduced significantly (Carter, 1978). The most important sunflower pathogens that are destructive under favourable conditions are *Plasmopara halstedii* [(Farlow) Berlese and de Toni], *Verticillium albo-atrum* (Reinke and Berthold) and *Sclerotinia sclerotiorum* [(Lib.) de Bary]. *Plasmopara halstedii* causing downy mildew, and infects plants in the seedling stage under moist and cool conditions and results in damping-off and seedling blight, where seeds may be killed before or soon after emergence. *Verticillium albo-atrum* causes wilting of sunflower which is evident near or at flowering time. The symptoms begin on the lower leaves and progress to the upper leaves (Carter, 1978). *Sclerotinia sclerotiorum* is a major pathogen of sunflower that infects roots, stems, and flower heads causing wilting, head rot and stem rot (Neergaard, 1977).

The genus *Alternaria* has been recorded to be a serious pathogen of sunflower causing a reduction in yield (Ojiambo *et al.*, 1998; Calvet *et al.*, 2005). *Alternaria* spp. are known to infect all parts of the plant viz., leaf, petiole, stem, all flower parts, and seeds (Kim and Mathur, 2006). The damage and effect caused by *Alternaria* spp. on the sunflower crop is that seedlings germinated from infected seed are often blighted (Suryanarayana, 1978).

2.2. The Pathogen: The Genus *Alternaria* Nees

2.2.1. Introduction

Alternaria Nees is a cosmopolitan fungal genus that includes saprophytic, endophytic and pathogenic species (Peever *et al.*, 2004). The genus *Alternaria* includes nearly 100 species that occur worldwide in a variety of habitats causing severe leaf and stem spots resulting in premature defoliation and stem breakage, as well as seedling blight (Carson, 1985; Jeffrey *et al.*, 1985; Pryor and Gilbertson, 2000). There are 36 known seed-borne *Alternaria* species, which might suggest that practically all species pathogenic to foliage also infect seeds (Rotem, 1994). The most common *Alternaria* species associated with sunflower plant leaf blight is *A. helianthi* (Hanford) Tubaki and Nishihara (Prasad *et al.*, 2008). Other common *Alternaria* species reported on sunflower include, *A. alternata* (Fries) Keissler, *A. zinniae* Ellis, *A. helianthicola* (Lapagodi and Thanassoulopoulos, 1996; 1998) and *A. protenta* Simmons (Cho and Yu, 2000).

2.2.2. History and nomenclature

Kingdom:	Fungi
Subkingdom:	Eumycotera
Phylum:	Ascomycota
Class:	Dothideomycetes
Order:	Pleosporales
Family:	Pleosporaceae
Genus:	*Alternaria*

The genus *Alternaria* was established in 1817 with *A. alternata* (*A. tenuis*) as the type isolate (Thomma, 2003). The genus was classified into the division of mitosporic fungi of the phylum

Fungi Imperfecti because of an absence of a known sexual stage of most of *Alternaria* species (Thomma, 2003). Thus, many *Alternaria* spp. are likely to be haploid fungi existing in a vegetative phase, reproducing asexually, and would be expected to have a high level of clonalityand this would display a low genotypic variation (Bock *et al.*, 2002; Guo *et al.*, 2004).

Alternaria are divided into three major sections or groups according to their catenation i.e. *Longicatenatae*, in which conidia appear in long chains of about 10 spores or more, as in *A. alternata*; *Brevicatenatae* which forms short chains, usually of three to five spores, as in *A. tenuissima;* and *Noncatenatae* which form solitary spores that may be beakless (*A. helianthi*), but may comprise species that have long beaks (*A. solani*) (Rotem, 1994; Chuo and Wu, 2002).

2.2.3. Morphology and cultural characteristics

Many small-spored *Alternaria* species produce small spores (40-70 x 12-20 µm) aggregating in branching chains with morphological characteristics that overlap those of *A. alternata*; the most commonly known are *A. tenuissima* (Fries) Wiltshire, *A. arborescens* E.G. Simmons and *A. infectoria* E.G. Simmons. Thus, the small-spored *Alternaria* are often misidentified (Pryor and Michailides, 2001). To allow characterization and identification these *Alternaria* spp., Simmons (1993) introduced the three dimensional sporulation patterns as a means of sorting out small-spored isolates from Asian pears (*Pyrus serotina* L.) into groups.

Sporulation of *Alternaria* species is affected by conditions such as lighting conditions, type media and temperature. *Alternaria* sporulation occurs in two phases. In the first phase, light enhances the formation of conidiophores and vegetative growth of the hyphae, but it inhibits conidial formation. In the second phase, the conidia are formed in darkness (Rotem, 1994; Pryor and Michailides, 2001). Light inhibits sporulation when the temperatures are relatively high, but not when temperature is lower (Masangkay *et al.*, 2000). The use of rich media promotes vegetative growth whilst restricting sporulation and thus reveals more variation in colour and texture colour of small-spored *Alternaria* spp. In contrast, for characterization of sporulation habit, nutritiously weak media and lighted incubation should be used (Pryor and Michailides, 2001).

The conidia of *A. helianthi* are light grey to grey on short conidiophores (Mathur and Kongsdal, 2003). The conidia are solitary, non-beaked, borne of simple unbranched conidiophores, cylindrical to elongate elliptic, yellowish brown septate with 3-10 transverse septa, with constricted septa, rounded at both ends and are 40-120µm x 15-28µm (average 100.6µm x 25.5µm) in size (Mirza*et al.*, 1984).

Alternaria alternata produces branching chains of small to medium-size, short-beaked conidia. The conidia may reach full size in a range of about 40-70µm x 12-20µm, although some species of the group do not reach such maximum conidium sizes in culture. *Alternaria alternata* conidia do not exceed 50µm in culture (Simmons, 1995). The conidiophores tends to branch or become successfully geniculate near the substrate surface and to initiate 2-8 chains that develop into a cluster of about 50-75 conidia (Fig. 2.1 A). Morphologically similar single conidia of Alternata species-group specimens may give rise in culture to colonies that are dissimilar in some macroscopic factor of growth rate, zonation, mycelium density, apparent colour, or timing and density of sporulation (Simmons, 1995). The conidia appear olivaceous, dull grey-green-brown and later exhibit pale to moderate shades of a yellowish to golden brown. Radial growth rate of typical *A. alternata* colonies is about 5mm/day when grown on potato carrot agar (PCA) at 22°C under a 10/14 hour, cool-white fluorescent light/dark cycle. Concentric rings of alternating dense and moderate sporulation are obvious on all media. These indicate increments of radial growth during successive exposures to daily light and dark cycles (Simmons, 1995).

Cultures of *Alternaria infectoria* sporulate as open, busy clumps of geniculate and branching secondary produced and separated by conidia (Fig. 2.1 B). The primary conidiophore may remain simple or may become elongate. It originates as a morphologically distinct branch of an aerial hypha. The length of the secondary conidiophores can be of any length up to 125 µm. The pattern of prominent secondary conidiophore elaboration and interspersed conidia is a conspicuous character of the *A. infectoria* group. Cultural characteristics of all *Alternaria* cultures are concentric rings of alternating dense and moderate sporulation on media (Simmons, 1994). Rao and Rajagopalan (1977) described *A. helianthicola* grown on PDA as profusely branched with abundant sporulation in 7 to 10 days. The conidiophores were often branched, septate and difficult to distinguish from mycelium. Conidia were golden yellow or dark brown,

ellipsoidal, 2 to 10 septae and constricted at septation. The length of the conidia inclusive of the beak was 66.5µm (29µm to 91.5µm) and the width 15.6 µm (7.25µm to 21.75µm), the conidia length alone ranged from 20µm to 40µm, whereas the beak length alone ranged from 45.5µm (29µm to 72.5µm) in length. Longitudinal septae were present in some conidia.

Alternaria arborescens was formally known as *A. alternata* f.sp. *lycopersici* (Pryor and Michailides, 2001), thus the *Alternaria arborescens* species-group has a similar three dimensional sporulation pattern to the *Alternaria alternata* species-group (Fig. 2.1 C) except that *A. arborescens* has longer conidiophores (Simmons, 1990). *Alternaria tenuissima* conidia are awl-shaped or have a narrowly tapered upper half; only transverse septa are present and they do not exceed 50 x 8 µm and have short apical conidiophores (Fig. 2.1 D). The conidia are medium golden brown in colour (Simmons, 1994).

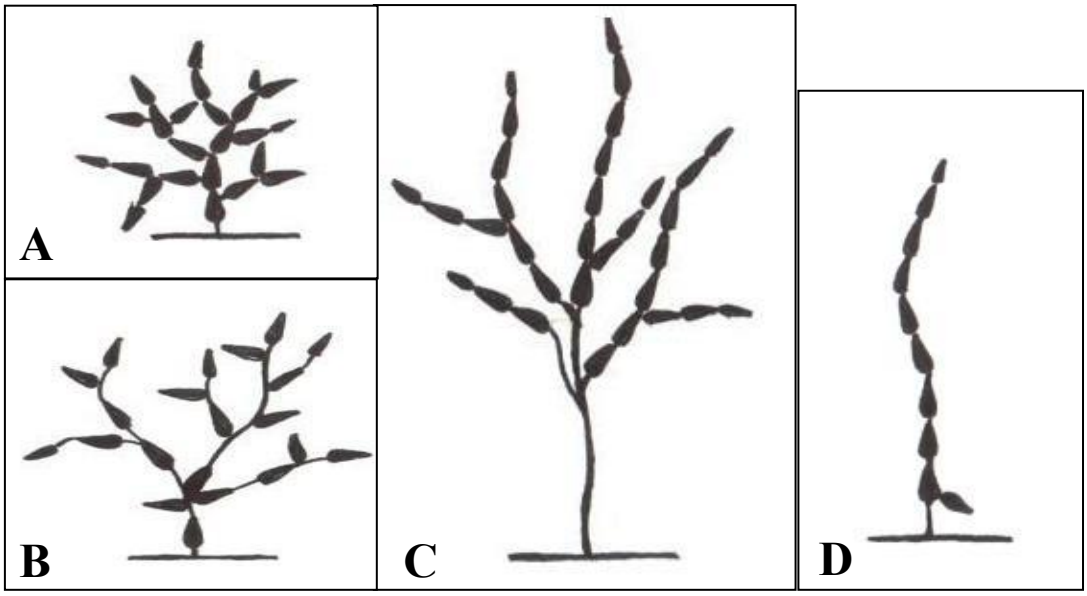

Figure 2.1: (A) *A. alternata*, (B) *A. infectoria*, (C) *A. arborescens* (D) *A. tenuissima*. (Simmons, 1994 and 1995).

2.2.4. Molecular characterisation

With the advancement of molecular techniques, several studies have examined taxonomic relationships amongst small-spored *Alternaria* species using a variety of methods in attempt to establish a consensus with classical morphological based identification. Molecular techniques

that have been used to determine the phylogenetic relationship between *Alternaria* species include restriction fragment length polymorphism (RFLP) (Pryor and Michaliades, 2001), (RAPD), internal transcribed spacer regions (ITS) of the ribosomal DNA (Konstantinova *et al.*, 2002; Quayyum *et al.*, 2005), the house keeping β-tubulin gene (Quayyum *et al.*, 2005), *endo*-polygalacturonase (*endo*-PG), glyceraldehyde 3-phosphate dehydrogenase (*gpd*), intergenic spacer (IGS) region of rDNA (Hong, 2005) and the mitochondrial small subunit (mtSSU) (Park *et al.*, 2008).

The ITS region cannot be used to characterize *Alternaria* species as it shows polymorphism between various small species such as *A. alternata, A. citri, A. mali,* and *A. longipes* (Kusaba and Tsuge, 1995; Peever *et al.*, 2004). β-tubulin and several other regions failed to differentiate between these isolates (Akimitsu *et al.*, 2003). Differentiation of the small-spored species has been difficult due to a lack of variation in nuclear ribosomal ITS and β-tubulin sequences (Peever *et al.*, 2004).

On the contrary to rDNA ITS and β-tubulin sequences, Quayyum *et al.*(2005) reported that the ITS region and the β-tubulin gene have been shown to be useful in separating morphologically similar *Alternaria* species, particularly those *Alternaria* species producing large and small conidia (Quayyum *et al.*, 2005). RAPD-PCR also provedthat there is great variation among the *Alternaria* species (McKay, 1999).

2.2.5. Hosts

Plant pathogenic *Alternaria* species infect a wide range of economically important plants such as tangerine (*Citrus reticulate* Blanco), apple (*Malus domestica* Borkh.), pear (*Pyrus pyrifolia* (Burm. f.) Nakai), tomato (*Lycopersicon esculentum* Mill.), potato (*Solanum tuberosum* L.), linseed (*Linum usitatissimum* L.), carrots (*Daucus carota* L.)and many others (McKay, 1999; Konstantinova *et al.*, 2001; Peever, 2004).

2.2.6. Geographical distribution

Infectious diseases are distributed geographically according to climatic conditions (Rotem, 1994). *Alternaria* species are ubiquitous and are highly resistant to adverse weather, because they can develop under a wide range of temperatures and can utilize locally available sources of moisture (Green and Bailey, 2000). Alternaria leaf blight of sunflower was first described as *Helminsthosporium helianthi* (Hansf.) in 1943 by Hansford as a cause of a blackish brown zonate spot on the leaves and later renamed to *A. helianthi* by Tubaki and Nishihara in 1969 (Allen*et al.,* 1983).Today various *Alternaria* species causing leaf spot on sunflower have been reported in almost all the parts of the world that grow sunflower (Mukhtar, 2009).

Africa:	Uganda	(Kong *et al.*, 1995)
	South Africa	(Westhuizen and Holtzhausen, 1980)
Asia:	India	(Prasad *et al.*, 2009)
	Korea	(Cho and Yo, 2000)
	Pakistan	(Nahar *et al.*, 2005)
	Thailand	(Prathuangwong *et al.*, 1991)
	Taiwan	(Wu and Wu, 2003)
North America:	U.S.A.	(Abbas *et al.*, 1995)
South America:	Brazil	(Calvet *et al.*, 2005)
Australia:	Australia	(Allen *et al.*, 1982)
Europe:	Greece	(Lagopodi and Thanasoulopoulos, 1998)

2.2.7. Symptoms

The Alternaria leaf blight diseases are known to infect all aerial parts of the plant such as the leaf, petiole, stem, floral parts and seeds. The symptoms caused by *A. helianthi* normally appear as dark brown, oval to circular spots with a pale margin and yellow halo. Spots are found on the leaves, stems, seeds, petioles, sepals and petals. The lesions become irregular by coalescing, leading to blight and defoliation and death of the plant (Cho and Yu, 2000). The destruction of the leaf and its organelles is caused by enzymatic reactions or by toxic metabolites formed by the

fungus during the infection period (Calvet *et al.*, 2005).If seed-borne, the *Alternaria* species can attack the seedling once the seed has germinated (Thomma, 2003).

Rao and Rajagopalan (1977) stated that the symptoms caused by *A. helianthicola* were seen initially as small, chlorotic spots with a brown centre on the leaves, the spots turn dark brown and are somewhat circular with yellow halos measuring about 3-5 mm in diameter after 10 days. The lesions later coalesce forming irregular dark patches leading to severe blighting, drying up of leaves and defoliation by 20 days. Leaf spot disease caused by *A. alternata* begins as zonate spots on basal leaves and progresses to upper leaves. Spots eventually coalesce, and the infected leaves dry up and cause premature defoliation (Ojiambo *et al.*, 1998). The fungus attacks flower heads causing head and seed rot (Lagopodi and Thanassoulopoulos, 1998). Infection of the seeds causes various biochemical changes. The fungus invades the embryo and the endosperm and reduces seed germination (Lagopodi and Thanassoulopoulos, 1998).

2.2.8. Toxins

Alternaria species produce host specific and non-host-specific toxins. *Alternaria helianthi* produces host specific toxin such as deoxyradicinin and 3-epideoxyradicinol (Robeson and Strobel, 1985), whereas *Alternaria arborescens* and *A. tenuissima* produces alternariol, alternariol monomethyl ether, altenuene, altertoxin I and tenuazonic acid. *Alternaria infectoria* produces alternariol monomethyl ether tenuazonic acid (Rotem, 1994; Andersen *et al.*, 2002). Different species of *Alternaria* produce different host-specific toxins according to their host-pathogen interaction and most of these toxins have been reported from the fungi identified as *A. alternata* (Andersen *et al.*, 2002; Thomma, 2003). Host specific toxins produced by *A. alternata* include AAL-toxin. This toxin closely resembles the mycotoxin Fumonisin B1 that was identified as *Fusarium moniliforme* toxin, and is also produced by AAL-toxin producing *A. alternata* species (Chen *et al*., 1992; Thomma 2003). However, Pryor and Michailides (2001) discovered that host specific toxins may not be involved in pathogenicity when they isolated *A. alternata, A. tenuissima* and *A. arborescens* from pistachio (*Pistacia vera* L.) which were not originally recovered from this plant.

The mode of action of the non-host-specific toxins is to degrade pectic polymers in cell walls during infection (Timmer *et al.*, 2003), causing spots and chlorosis in many plant species (Agrios, 2005), whereas host-specific toxins cause electrolyte leakage and necroses by affecting the plasma membrane by increasing NADH oxidation, acting on the chloroplast, inducing swelling and other morphological modifications of the mitochondria (Rotem, 1994; Thomma, 2003).

2.2.9. *Alternaria* allergens

Apart from being phytotoxic, some *Alternaria* toxins are toxic to mammals and birds (mycotoxins). Fungal spores can also have a serious detrimental effect on human health, trigger respiratory diseases and allergenic processes (Rodriguez *et al.*, 2005). *Alternaria* species impact lung epithelial cells that provide innate immune defense against airborne environmental constituents (Boitano *et al.*, 2011). The genus *Alternaria* has been cited in up to 30% of the respiratory allergies (Escuredo *et al.*, 2010). There are allegations of *Alternaria* species to be considered a potential cause of cancer (Konstantinova *et al.*, 2002).

2.2.10. Epidemiology and disease cycle

Alternaria pathogens are generally disseminated predominantly by seeds. The seed is infected outside the embryo. During germination the pathogen is carried either passively on the cotyledons, or on the seed coat or it is transmitted to the young plant by air currents and rain splashes (Rotem, 1994).The seed may be infected either systemically from the mother plant or from the outside. During systemic infections, the fungus grows into the ovule and later develops from the seed further into the seedling. *Alternaria lolii-temulenti* Agostini in *Lolium temulentum* L. has been confirmed to be a symptomless systemic invader (Neergaard, 1977). The sunflower crop may be infected from the outside by conidia being disseminated by wind to young florets of healthy heads. In some cases the pathogen may be found contaminating the seed surface (Suryanarayana, 1978).

All *Alternaria* epidemics generally start when the plants begin flowering and reach their maximum intensity during plant senescence (Rotem, 1994). The factors that contribute to epidemics of Alternaria blight include an increase in inoculum, environmental conditions that

favour an elevated level of spore deposition, and predisposition to wounds (Pleysier *et al.*, 2006). Older tissue is more susceptible to *Alternaria* infection causing premature leaf senescence. Under favourable conditions, the leaf spots spread progressively to the upper leaves (Prasad*et al.*, 2009). For diseases caused by *Alternaria* spp. susceptibility increases as plant tissues matures (Green and Bailey, 2000).

Alternaria leaf spot on various crops is usually favoured by temperatures of 25 to 30°C and 12 hours of leaf wetness (Allen *et al.*, 1982; Reis *et al.*, 2006; Prasad 2009). With optimum temperatures and small amounts of leaf wetness, infection can occur within 4 to 8 hours, but usually 10 to 12 hours are needed for substantial infection and results in a high disease incidence. As temperatures decline, longer wetting periods are needed for infection to occur (Timmer *et al.*, 2003; Reis *et al.*, 2006). Alternaria blight development can still occur with minimum temperatures of 14-15°C and a relative humidity of above 65% (Sangeetha and Siddaramaiah, 2007). Free water is essential for germination and infection by *Alternaria* spp. (Rotem, 1994), but Green and Bailey, (2000) reported that free water is optimal, but not essential, for the infection of Canada thistle (*Cirsium arvense* L.) by *A. cirsinoxia* (Simmons and Mortensen). Under favourable conditions, the conidia of most *Alternaria* species germinate within 3 hours (Quayyum *et al.*, 2005). *Alternaria* spp. often produce more than one germ tube, and penetrate the leaf cells directly through walls or stomata. Stomatal penetration is often by chance and hyphae often grow over the stomata without penetration (Slavov *et al.*, 2004).

The disease cycle of most *Alternaria* spp. is simple because of their unknown sexual stage (Guo *et al.*, 2004). Figure 2.2 depicts a generalised disease cycle of an *Alternaria* species. The fungus overwinters or survives as spores or mycelium on decaying plant debris or as a latent quiescent infection in seeds (Thomma, 2003). If the fungus is carried with the seed, it may attack the seedling, usually after emergence and cause seedling blight, damping-off or stem lesions. Conidial production and release are triggered by heavy dews and frequent rains and high temperatures, and blown from infected plants or debris (Reis *et al.*, 2006). The germinating spores penetrate susceptible tissue directly, or through natural openings or through wounds and soon produce new conidia that spread by wind or splashing rain. *Alternaria* diseases are more

prevalent on older, senescing tissues, especially on plants growing poorly because of some kind of stress (Agrios, 2005).

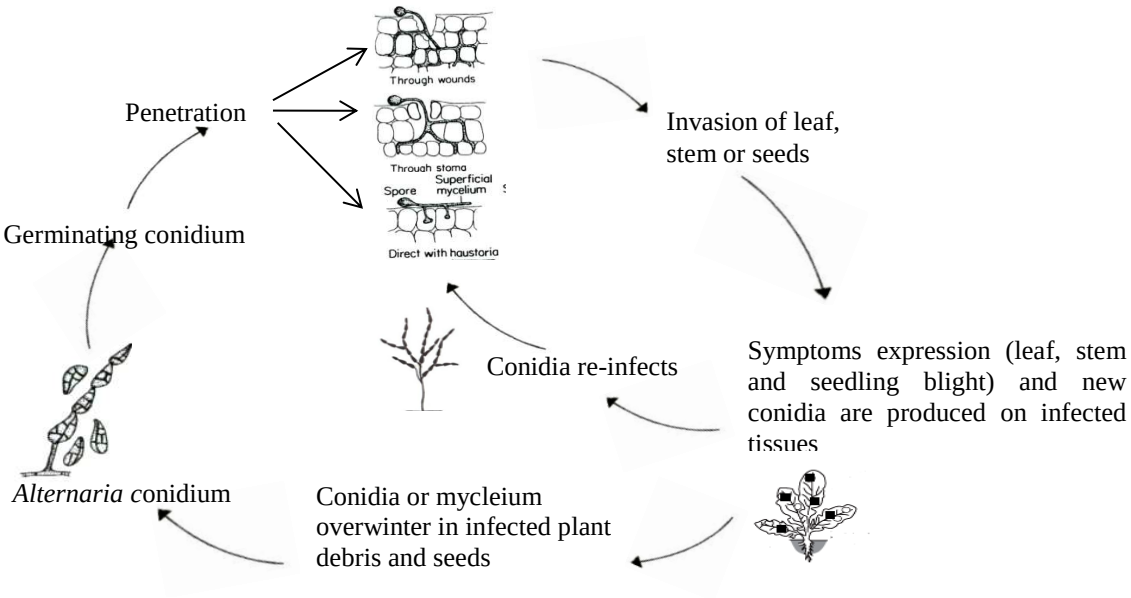

Figure 2.2: The generalised disease cycle of an *Alternaria* species. (Agrios, 2005).

2.3. Seed Quality

The definition of seed quality is broad, comprising five properties of seeds namely purity, storage, viability, vigour and health (ISTA, 2012).

2.3.1. Seed purity and moisture content

Seed purity refers to both physically and genetically pure seeds. Physical purity implies the absence of foreign material such as stones, twigs and weed seeds; whereas genetic purity implies that the seed lot contains seeds with the genuine, known characteristics of that particular cultivar (McDonald, 1998; ISTA, 2012). Moisture content is a parameter that needs to be assessed to gain insight into storage ability of a seed lot. The moisture content test must be done as soon as the seeds arrive in the laboratory because the seeds will either gain or lose moisture when they are exposed to the ambient air. Seeds with high moisture content have a poor storage potential (Joao and Lovato, 1999; ISTA, 2012).

2.3.2. Seed viability tests

Seed viability is the ability of a seed to germinate and produce a new plant. Germination tests are seed viability tests that are done to determine the maximum germination potential of a seed lot, which can be used to compare the quality of different lots and estimate the field planting value (ISTA, 2012). Seeds with a germination percentage greater than 80% can be used by the scientist for his subsequent experiments or by the farmer to cultivate his crop. Seeds can be germinated in various media such as paper, sand and organic growing media. Replicates of 100 seeds are normally used, spaced sufficiently far apart on the growing media to avoid the effect of the adjacent seeds on seedling development (ISTA, 2012).

The tetrazolium test aims to determine whether tissues are alive and have the potential to germinate under optimal temperatures. The tetrazolium test can be used as an alternative test for the germination test if there is not sufficient time (7 to 10 days) to conduct the germination test. Triphenyltetrazolium chloride (TTC) is a colourless chemical that reacts with living tissue and stains it red. Thus, living tissue in the seed can be distinguished from non-living tissue within 12 to 24 hours after application of TTC (ISTA, 2012).

2.3.3. Seed vigour

Seed viability and vigour directly affect the performance of seeds planted to regenerate a crop. Seed vigour testing does not only measure the percentage of viable seed in a sample but also reflects the ability of those seeds to produce normal seedlings under less optimum conditions, similar to those which may occur in the field (ISTA, 2012). Factors known to influence seed vigour include genotype, the nutrition and growth conditions of the mother plant, the physiological maturity of the seed at harvest, physical handling of the seed during processing, seed moisture content and temperature during storage (Saranga *et al.*, 1998).

Seed vigour is the sum of those properties of the seed that determine the potential level of activity and performance of the seed or seed lots during germination and emergence (TeKroney and Egli, 1990). Several vigour tests which assess performance of seed lots under stress conditions have been developed viz. seed cold test, accelerated ageing and conductivity tests. The cold test is done by germinating seeds in wet soils and incubating them at 5 to 10°C for a

certain period. The principle of the cold test is to select for cultivars with the best ability to perform under cold wet soils for early spring (Van Waes, 1995).

The accelerated ageing test is done to stress seeds with high temperatures (40-45°C) and near 100% relative humidity for varying durations depending on the kind of seeds, after which the germination test is followed. The principle is that high vigour seeds are expected to tolerate high temperatures and relative humidity and still maintain the ability to produce normal seedlings in the germination tests (Hampton and TeKroney,1995). The electrolyte conductivity test measures the integrity of cell membranes, which is correlated to seed vigour. As seeds lose vigour, nutrients exude from their membranes and so low quality seeds would leak electrolytes such as amino acids while high quality seeds maintain the nutrients within well-structured membranes. Thus, electrolyte conductivity of the seeds can be measured (Prasil and Zamecnik, 1997; Bajji *et al.,* 2001).

2.3.4. Seed health

Seed health is the presence or absence of disease-causing microorganisms such as fungi, bacteria and viruses. Seed health is imperative because the diseases initially present on the seeds may give rise to progressive disease development in the field and reduce the commercial value of the crop (ISTA, 2012). The presence of microorganisms on seeds can be determined using the agar plate and blotter methods. Both these methods base their identification on morphological characters using a stereomicroscope. Seeds are surface sterilized and placed on the well-soaked filter paper (blotter method) or agar medium (agar plate method) and incubated for 7 days at 22°C under 12 hours alternating cycles of light and darkness (Mathur and Kongsdal, 2003).

Alternaria species may either be pathogenic or saprophytic (Pryor and Gilbertson, 2000; Peever *et al.,* 2004). Depending on the pathogenicity of the *Alternaria* species, the species may be viewed as merely a contaminant only being carried on the seed coat or as a pathogen that may penetrate the seed to invade and infect the seed coat, endosperm and embryo. Seeds are vectors that transmit disease to seedlings causing infections on cotyledons as they emerge from the soil (Rotem 1994). If an *Alternaria* pathogen is seed-borne, the fungus can attack the seedling once

the seed has germinated or may cause poor germination and seedling damping-off (McKay, 1999; Thomma, 2003).

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Chapter 3: Alternaria leaf spot disease on sunflower (*Helianthus annuus*) caused by *Alternaria helianthicola*

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A field trip was taken to sunflower (*Helianthus annuus* L.) fields in Greytown Northern KwaZulu-Natal in early March 2010 during the warm and rainy summer season. An *Alternaria* species was isolated from lesions of a leaf blight disease of sunflower. The symptoms were characterised by circular, brown lesions that were surrounded by a chlorotic halo. As time elapsed, the lesions coalesced, to form larger lesions. The species was identified as *A. helianthicola* based on the morphological characteristics of conidia, conidiophores and the three-dimensional sporulation pattern. Koch's postulates were completed when the *Alternaria* pathogen was re-isolated from inoculated greenhouse plants and produced cultures with identical morphological features as those originally isolated from sunflowers from KwaZulu-Natal. A disease severity index was compiled using symptoms from the pathogenicity test and Koch's postulates. *Alternaria helianthicola* has not previously been recorded on sunflower in South Africa.

Keywords: summer, *Helianthus annuus*, symptoms, *A. helianthicola*, Koch's postulates, disease severity index

3.1. Introduction

Alternaria leaf spot on sunflower (*Helianthus annuus* L.) is an important disease that can cause a significant reduction in crop yield (Allen *et al.*, 1982). *Alternaria helianthi* is one of the main causal agents of Alternaria leaf spot of sunflower (Cho and Yu, 2000). *A. helianthicola* was first recorded in India by Rao and Rajagopalan (1977) after causing severe leaf spots on sunflower crops. *Alternaria* species can affect the leaves and stems of sunflower plants during emergence and throughout the growing stages of the plant (Mirza *et al.,* 1984; Malone and Muskett, 1997). Symptoms are seen on the foliage as brown, oval to circular spots with a pale margin and yellow halo. In severe infections, lesions become irregular by coalescing, leading to blight and premature defoliation and death of plant. Spots are found mainly on the leaves, but are also seen on stems, petioles and sepals and petals (Calvet *et al.,* 2005). These symptoms negatively influence the photosynthetic process, by altering the chloroplast, which leads to chlorophyll degeneration and reduction of CO_2 fixation (Ojiambo *et al.,* 1998).

Another foliar pathogen on sunflower that causes similar but less severe leaf spot as the *Alternaria* spp. is Septoria leaf spot, caused by *Septoria helianthi* Ellis and Kellerman (Hamid and Jalauddin, 2007). Septoria leaf blight is destructive under conditions of abundant rainfall (Block, 2004). Symptoms caused by *S. helianthi* are initially seen as individual spots that coalesce to form yellow to dark brown irregular blotches (Hamid and Jalaluddin, 2007). Septoria leaf blight is less severe and damaging than Alternaria leaf blight (Carson, 1985).

Field trips to Northern KwaZulu-Nataland Potchefstroom during the period of 2010 to 2012, found a severe leaf spot disease in sunflower fields. The symptoms that were seen on the sunflower crop resembled those caused by *A. helianthi.* Epidemics of Alternaria blight of sunflowers are most common and severe in areas that experience extended periods of wet weather in the summer accompanied by mean and daily temperatures between 25 and 30°C (Allen *et al.,* 1982). The objective of this study was to determine the causal agent of the leaf spot disease on sunflower and compile a disease severity index of its symptoms.

3.2. Materials and Methods

3.2.1. Pathogen cultures

Alternaria leaf spot infected sunflower leaves were collected from a sunflower field in Greytown, Northern KwaZulu-Natal Province (S29°0456.54, E30°3601) during a field trip in 2010. Samples of the diseased material were kept in cold storage during transportation to the laboratory. The leaves were cut into 5 mm^2 blocks (containing partly infected and healthy parts) and surface sterilised in 1% (w/v) sodium hypochlorite for 3 min, rinsed twice in sterile distilled water and plated onto potato dextrose agar (PDA) (Merck) amended with 0.01% chloramphenicol. The plates were incubated at 25°C in a 12hour UV-light/12hour dark cycle for 3 days. The *Alternaria* isolates that grew from the infected leaves were transferred to new PDA plates amended with chloramphenicol and incubated at 25°C in a 12hour UV-light/12hour dark cycle for 14 days. Single spore isolations of the *Alternaria* species was prepared by pouring 4 ml of sterile distilled water on 14 days-old cultures. Two ml of the suspension was spread uniformly on PDA amended with chloramphenicol and incubated at 25°C for 24 hours. The Petri dishes were examined under a Zeiss light microscope to locate single isolated and germinated spores. The *Alternaria* isolate cultures were maintained as conidia frozen in 20% glycerol at -20°C and as mycelial blocks in double sterile distilled water at 4°C until needed for subsequent experiments, as all inoculations were done using this *Alternaria* isolate.

3.2.2. Morphological identification

The morphological identification of the pathogen was done using the standard cellophane technique preparation. A piece of clear cellophane tape was gently pressed onto the surface of a culture that was grown on PDA and incubated at 25°C under 12hourUV-light/12hour dark cycle for 14 days, and gently lifted and placed onto a glass slide. The morphology of the *Alternaria* isolates was observed and examined under a Zeiss light microscope without the use of a cover slip. The characteristics of the isolates were compared to those *Alternaria* species as described by Simmons (1990; Simmons and Roberts, 1993; 1994; 1995).

3.2.3. Plant material

Sunflower (*Helianthus annuus* L.) seeds (cultivar PAN 7351) were received from Pannar (PTY, LTD), Bapsfontein, South Africa. The seeds were surface sterilised with 0.5% sodium hypochlorite solution for 5 min and rinsed in sterile water for a minute. Five seeds were sown in each 12 cm diameter plastic pots containing pasteurised soil (light red, pH (H_2O): 6.6, pH (KCl): 6, sand: 85%, clay: 11%, silt: 4%, texture: loamy sand). The plants were maintained in a greenhouse at temperatures of 25 to 30°C and a relative humidity of 50-80% with a photoperiod of 16 hours and watered daily. The plants were left to grow until 4-weeks-old before use in the subsequent experiments.

3.2.4. Pathogenicity test and Koch's postulates

Sunflower plants grown as above were inoculated with a $4x10^5$ spores/ml conidial spore suspension of 2-weeks-old *Alternaria helianthicola* isolates. The spore suspensions were prepared by adding 5 ml of sterile distilled water to each Petri dish, and dislodging the conidia with a sterile hockey stick. Each suspension was poured in a beaker and amended with 0.05 µl Tween 20 (Merck). The solution was passed through cheesecloth to obtain spores. The spore concentration was determined using a haemocytometer. The conidial suspension was stirred to prevent clumping of conidia and inoculated immediately after preparation by spraying the leaves until run-off with an automatic aerosol sprayer. The plants were then covered with polyethylene bags to maintain a high relative humidity (>95%) within the surrounding area of the plants and incubated in the greenhouse at 25°C. The evaluation of the infection was done over a period of 7 days. The isolate causing the same leaf spot lesions as initially observed on the farms in KwaZulu-Natal was re-isolated on PDA. The pathogen was then identified morphologically using Simmons (1990; Simmons and Roberts,1993; 1994; 1995). A disease severity index was compiled using symptoms from the pathogenicity test. A representative culture was submitted to the Agricultural Research Council – Plant Protection Research Institute, Mycology Unit (ARC-PPRI) for the confirmation of the morphological identification.

3.3. Results

Figure 3.1 depicts the Alternaria leaf spot symptoms on sunflower plants as seen in sunflower fields in Greytown. The symptoms that were seen on the foliage were brown, oval to circular spots with a pale margin and yellow halo. In severe infections, lesions become irregular by coalescing (red encircled area in Fig. 3.1), leading to blight and premature defoliation and death of the plant. Spots were found mainly on the leaves, but the spots were also seen on other plant parts viz. stems, petioles, sepals and petals.

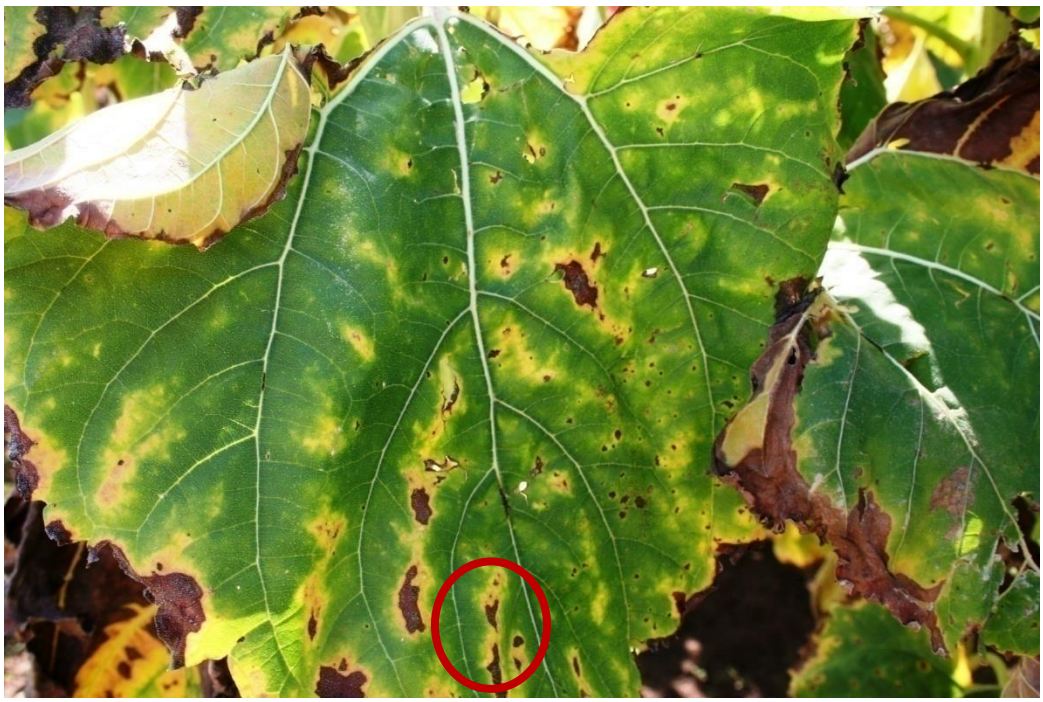

Figure 3.1: Alternarialeaf spot symptoms seen in sunflower (*Helianthus annuus*) fields in Greytown. Red encircled area indicates coalescence of lesions.

3.3.1. Morphological identification

The *Alternaria* isolate was characterized by long secondary conidiophores that developed from the conidia. The secondary conidiophores were branched and difficult to distinguish from mycelium, often with acropleurogenous conidia that resulted in complex branching that were observed as bushy (Fig. 3.2). The conidia were mid-golden brown in colour and polymorphous. The conidia appeared to be oval or elliptical and often obclavate. The conidia had 3 to 7 transverse septa; some were dictyoconidium with oblique septa. The conidia were slightly constricted at septation. The length of the conidia ranged from 13 to 25µm. The width of the conidia was 12 to 15µm. The colony growth pattern was circular with alternating circles due to the alternating 12 hours light and darkness regime (Fig. 3.5). The colony texture was woolly. Although the isolate had similar characteristics than *A. infectoria* (Simmons 1990; 1993; 1994; 1995), it differs in terms of the conidia surface ornamentation. The isolate in the present study had punctate conidia, whereas those of *A. infectoria* are smooth. The culture was identified by the Agricultural Research Council – Plant Protection Research Institute, Mycology Unit (ARC-PPRI), Pretoria, as *Alternaria helianthicola* (PPRI 11433).

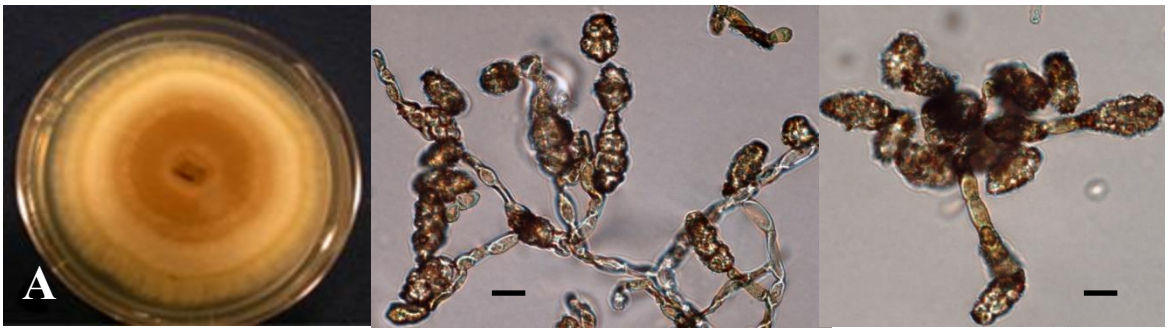

Figure 3.2: Culture and sporulation structures of *Alternaria helianthicola* isolated from foliar lesions of sunflower (*Helianthus annuus* L.). Each bar represents 10 µm. Light microscope of 40x magnification was used.

3.3.2. Pathogenicity test and Koch's postulates

The symptoms on the inoculated plants were visible from 2 days post inoculation. Lesions on the leaves were circular to irregular with a light brown centre (Fig. 3.3). The lesions were seen both on the inner part of the leaf and on the leaf margin and leaf tip. After a period of seven to fourteen days the leaves shrivelled and dried up (Fig. 3.4). During the evaluation, the symptoms were seen also on older leaves. A point to note is that a few of the control plants that were inoculated with sterile distilled water developed mild symptoms (disease rating of less than 1, as described in Table 3.1) Alternaria leaf spot symptoms. However, all the sunflower plants inoculated with *Alternaria helianthicola* spore suspension isolated from the leaves developed disease symptoms similar to the symptoms observed in the sunflower fields. Koch's postulates were thus completed when the *A. helianthicola* was re-isolated from the greenhouse plants and produced cultures with identical morphological features as those originally isolated from KwaZulu-Natal.

Figure 3.3: The initial symptoms of *Alternaria helianthicola* on sunflower (*Helianthus annuus* L.) plants formed within four days after inoculation during a pathogenicity test. Leaf symptoms either began at the leaf edges or in the centre of the leaf.

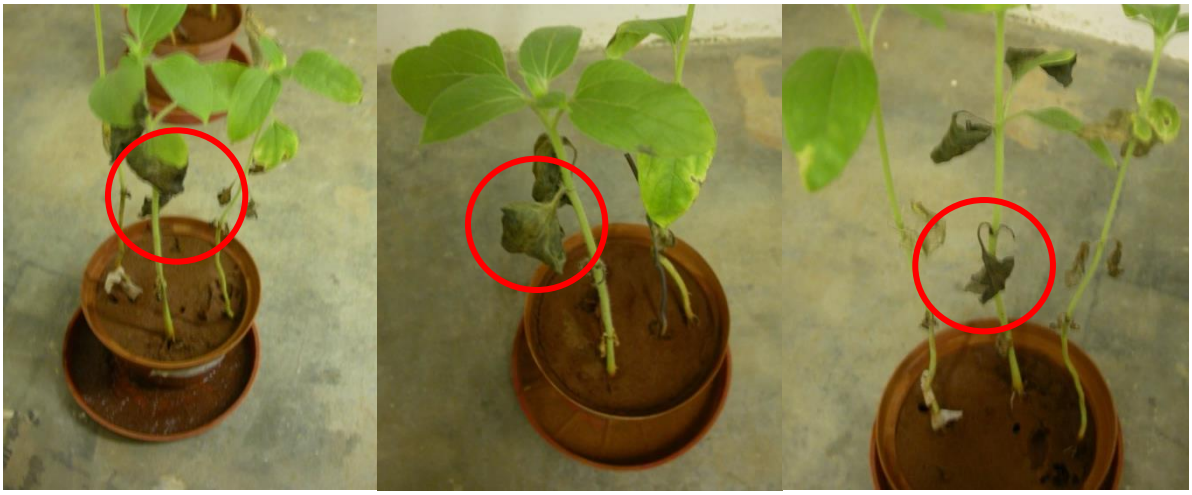

Figure 3.4: The symptoms of *Alternaria helianthicola* on sunflower (*Helianthus annuus* L.) formed within 10 to 14 days during a pathogenicity test.

A disease severity index compiled using symptoms from the pathogenicity test is presented in Figure 3.5 and Table 3.1. This index will be used in the upcoming chapters to determine the disease severity of this pathogen under certain environmental conditions.

Figure 3.5: The development of leaf spot symptoms on leaves of sunflower (*Helianthus annuus*). (A) An asymptomatic sunflower leaf, (B) Often symptoms begin as circular lesions on the inner part of the leaves or on the leaf margins. (C) As the time elapsed, disease the lesions became enlarged in size, (D) and as more lesions were formed and they eventually coalesce to form larger irregular lesions, (E) to cause premature shrivelling of the leaves. The lesions are always surrounded by a chlorotic halo as soon as they are formed.

Table 3.1: Disease severity index used to record *Alternaria helianthicola* on sunflower (*Helianthus annuus*)

Disease severity	**Disease symptoms on sunflower leaves**
0	No visible expression of disease (Fig. 3.4 A)
1	Small greyish-brown lesions (± 1-2mm), surrounded by a yellow halo, in the inner part of the leaf or on the leaf margin or 1 to 25% leaf infection (Fig. 3.4 B).
2	Lesions enlarge to ± 8mm in diameter and become necrotic or 26 to 50% leaf infection (Fig. 3.4 C).
3	Multiple lesions that coalesce and enlarge to about ± 13mm in diameter or 51 to 75% leaf infection (Fig. 3.4 D).
4	Defoliation of the leaf, leaf would be severely damaged or 76 to 100% leaf infection (Fig. 3.4 E).

3.4. Discussion

Alternaria leaf blight of sunflower is mostly caused by *A. helianthi* (Sackston, 1981; Lagapodi and Thanassoulopoulos, 1998). *A. helianthi* is characterised by the absence of mycelia, solitary and non-beaked conidia that are normally cylindrical to obclavate with rounded ends. The conidia have 2 to12 septa and its length varies from 45 to 145 µm and its width ranges from 10 to 30 µm (Mirza *et al*., 1984; Mathur and Kongsdal, 2003). In this study, the fungus that was isolated from the leaf spot on sunflower was identified morphologically as *A. helianthicola.*

The main characteristic observed in the *A. helianthicola* cultures was secondary conidiophores that were indistinguishable to mycelium and developed from acropleurogenous oval to elliptical conidia that resulted in complex branching that were observed as bushy. Similarly, Rao and Rajagopalan (1977) described *A. helianthicola* grown on PDA as profusely branched with abundant sporulation in 7 to 10 days. The conidiophores were often branched, septate and difficult to distinguish from mycelium. Conidia were golden yellow or dark brown, ellipsoidal, 2

to 10 septae and constricted at septation. The length of the conidia inclusive of the beak was 66.5 µm (29 to 91.5) and the width 15.6 µm (7.25 to 21.75), the conidia length alone ranged from 20 to 40 µm, whereas the beak length alone ranged from 45.5 µm (29 to 72.5) in length. Longitudinal septae were present in some conidia.

Alternaria helianthicola belongs to the Infectoria-species group and Simmons (1994) described the Infectoria species group as cultures that sporulate as open, busy clumps of geniculate and branching secondary conidiophores produced and separated by conidia. The length of the secondary conidiophores can be of any length up to 125 µm. The pattern of prominent secondary conidiophores elaboration and interspersed conidia is a conspicuous character of the *A. infectoria* group (Simmons, 1994). The Infectoria species-group is one of fourteen groups of the small-spored *Alternaria.* Other small-spored *Alternaria* groups include the Alternata species-group, Tenuissima species-group and Arborescens species-group (Simmons, 1995).

The small-spored *Alternaria* spp. have morphological characteristics that overlap in terms of size, shape and conidia pigmentation to those of *A. alternata*. Thus, the small-spored *Alternaria* are often misidentified (Pryor and Michailides, 2001). *Alternaria* morphology varies under different cultural conditions, which can be misleading in descriptive and comparative studies of small-spored species (Andersen *et al.,* 2002). To confirm the identity of this pathogen, additional identification methods using molecular techniques will be used in Chapter 5 of this research.

Artificial inoculation of the sunflower plant in the greenhouse produced similar leaf spot disease symptoms to those in the field, and re-isolation from the inoculated leaves proved that *A. helianthicola* was the causal agent of leaf spot and blight of sunflower. The symptoms began as circular lesions on the inner part of the leaves or on the leaf margins. As time elapsed, more lesions were formed and eventually coalesced forming larger irregular lesions that caused premature shrivelling of the leaves. The lesions were surrounded by a chlorotic halo. Rao and Rajagopalan (1977) described the symptoms initially as small, chlorotic spots with a brown centre on the leaves, the spots turn dark brown and are somewhat circular with yellow halos measuring about 3-5 mm in diameter after 10 days. The lesions later coalesce forming irregular dark patches leading to severe blighting, drying up of leaves and defoliation by 20 days. A few

of the control plants that were inoculated with sterile distilled water developed mild Alternaria leaf spot symptoms which may indicate that seed lots received from various seed companies may be infected with *Alternaria* species. This is further discussed in Chapter 4 of this research.

Alternaria helianthicola was first described in India by Rao and Rajagopalan (1977) and later in Taiwan (Wu and Wu, 2003) and Croatia (Vrandecic *et al.*, 2011). This is the first report of *A. helianthicola* as a plant pathogen in South Africa. Most *Alternaria* species are regarded as saprophytic endophytes or weak pathogens (Thomma, 2003; Gat *et al.*, 2012), but the results of this study show that the *A. helianthicola* can be as damaging as *A. helianthi.* In conclusion, *A. helianthicola* was identified as a serious pathogen of sunflower and a disease severity rating scale was developed for future research.

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Chapter 4: The influence of seed infection by *Alternaria* spp. on sunflower (*Helianthus annuus* L.) seed germination

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Sunflower (*Helianthus annuus* L.) is only propagated by seed. Standard germination and seed health tests were conducted for thirteen sunflower seed lots from various parts of South Africa to determine whether seed infection by *Alternaria* spp. had an effect on germination on those seed lots. Germination percentages ranged from 60 to 94%. Germination was found to be influenced by the severity of seed infection, although the correlation was a fairly weak (56%).Seeds placed onto potato dextrose agar (PDA)and incubated at 25°C under 12 hours of alternating dark and UV light cycles showed that the various seed lots had *Alternaria* spp. infection ranging from 16 to 98% caused by various small-spored *Alternaria*. Germination tests showed that the germinated seedlings of the various seed lots had seedling blight. Seed component plating tests showed that the *Alternaria* species were more prevalent in the embryo and cotyledon than on the seed coats.

Keywords: seed health, small-spored *Alternaria*, seedling blight, germination, component plating test

4.1. Introduction

Helianthus annuus L. (sunflower) is the most produced edible oil crop in South Africa. The quantity of the sunflower oil represents about 82% of all edible oil produced in South Africa. The sunflower crop is propagated by seeds (Esterhuizen and Sindelar, 2010). It is recorded that more than 24 fungal species are seed-borne in the crop. The most common seed-borne fungi that have been reported on sunflower seed include *Verticillium, Sclerotium, Plasmopara, Aspergillus, Cladosporium, Curvularia, Dreschlera, Alternaria, Fusarium* and *Penicillium* (Neergaard, 1977; Nahar *et al.,* 2005).

Among the predominant *Alternaria* seed-borne pathogens of sunflower are *A. helianthi, A. zinniae, A. protenta* and *A. alternata* (Allen *et al.,* 1982; Prathuangwong *et al.,* 1991; Cho and Yu, 2000). Sackston (1981) reported that *A. helianthi* can cause seedling blight and head rot. *Alternaria* spp. are known to destroy up to 80% of sunflower yield (Allen *et al.,* 1983) and oil yield of up to 33% (Calvet *et al.,* 2005). Seed infection by *Alternaria* species can result in reduced seed production, reduced seed germination, reduced vigour and contamination of seeds (Pryor and Gilbertson, 2001; Prasad *et al.,* 2008). *Alternaria* species attack the embryo and endosperm causing various biochemical changes resulting in the degradation of seed components (Lagapodi and Thanassoulopoulos, 1998). The yield components most affected by Alternaria leaf blight are number of seeds per head; seed fill, kernel weight and oil content (Calvet *et al.,* 2005).

Sunflowers are susceptible to *A. helianthi* during the anthesis and seed development stages (Mesta, 2006). The disease initially appears during the seedling stage and especially on the more susceptible cotyledons (Bashan *et al*., 1991). In addition to yield losses caused by them, several mycotoxic or carcinogenic compounds harmful to plants and animals are also produced by these species (Andersen *et al.,* 2002).

Previous studies have shown that *Alternaria* species overwinter in naturally infected seeds and crop debris (Bashan *et al*., 1991, McKay *et al.* 1999). One of the greatest hazards in agriculture is sowing seeds that do not have the capacity to produce an abundant crop. Thus, the importance of seed testing is to minimise this risk by assessing the quality of seed before it is sown (ISTA,

2012). The objective of this study was to determine the percentage germination of thirteen sunflower seed lots collected from different farms in the various provinces of South Africa and to determine whether seed infection by *Alternaria* spp. had an effect on germination on those seed lots.

4.2.Materials and Methods

Thirteen seed lots of sunflower (*Helianthus annuus*) were received from South African seed companies including Pannar, Bapsfontein (PAN 7057, PAN7063 (CL), PAN7049, PAN 7351 and PAN 7050), Pannar, Greytown (PAN 7050 and PAN 7351), Senwes, Viljoenskroon (Nk ferti, Agsun s661 and Agsun s181), Senwes, Bloemfontein (A5671 and A8251) and Senwes, Ventersdorp (Agsun 8251). All seeds were untreated with fungicide seed treatments.

4.2.1. Standard germination test

The standard germination test was conducted in rolled paper towels at 25°C according to the rules of the International Seed Testing Association (2012), with the exception that prior to each germination test, sunflower seeds were surface sterilised with 1% hypochlorite for 5 min and rinsed with sterile distilled water. In this test, four layers of germination paper towels were used. For each seed lot, 50 seeds were placed on top of three layers of germination paper towels and then covered with the fourth layer of germination paper towel. The paper towels were moistened with 150 ml of distilled water. The seeds were loosely rolled in the four layers, placed in sealed polyethylene bags and placed in an upright position in an incubator at 25°C with an alternating 12 hour darkness and normal light regime. The first and second count of germination was made after four and 10 days. Germination tests were done as four replicates of 100 seeds. The experiment was repeated three times.

4.2.2. Isolation of *Alternaria* species from germinated seeds

Alternaria species were isolated from lesions of germinated sunflower seeds subsequent to the standard germination tests. The infected cotyledons were cut into 5 mm^2 blocks (containing part infected and healthy parts) and surface sterilised in 1% (w/v) sodium hypochlorite for 3 min, rinsed twice in sterile distilled water and plated onto PDA amended with 0.01%

chloramphenicol. The plates were incubated at 25°C in a 12 hour UV-light/12 hour dark cycle for 3 days. The *Alternaria* isolate that grew from the infected leaves was transferred to new PDA plates amended with chloramphenicol and incubated at 25°C in a 12 hour UV-light/12 hour dark cycle for 14 days. A single spore isolation of the *Alternaria* isolate was prepared by pouring 4 ml of sterile distilled water on a 14 days-old culture. Two ml of the suspension was spread uniformly on PDA amended with chloramphenicol and incubated at 25°C for 24 hours.

4.2.3. Agar plate seed health method

Two hundred seeds of each of the 13 sunflower seed lots were surface-disinfected with 1% sodium hypochlorite for 5 min, rinsed with sterile distilled water and placed on sterile paper towel in the laminar flow until dry. The seeds were placed onto potato dextrose agar medium (PDA) (Merck), with five seeds in each Petri dish. The plated seeds were incubated for 5-7 days at 25°C under 12 hours alternating cycles of ultraviolet (UV) light and darkness. The fungi that grew from seeds on the agar medium were examined and identified. Identification was based on colony characters and morphology of sporulating structures (Simmons, 1990, 1993 (Roberts), 1994, 1995; Mathur and Kongsdal, 2003) under a Zeiss light microscope. There were four replicates of 50 seeds. The experiment was repeated three times.

4.2.4. Seed component test using the agar plate method

One hundred seeds per cultivar were used for the detection of *Alternaria* spp. from separate parts of the sunflower seeds. Each seed sample was soaked in sterile distilled water for 7 to 8 hours at 25°C. After the seed coat had softened, the seeds were cut open to separate the cotyledon/embryo from the seed coat. The seed coat and cotyledon/embryo of the various sunflower cultivars were surface-disinfected with 1% sodium hypochlorite for 5 minutes and rinsed in sterile distilled water. The various seed components were placed onto PDA medium and the plated seed parts were incubated for 5-7 days at 25°C under 12 hours alternating cycles of UV light and darkness. The fungi that grew from seed parts on the agar medium were examined and identified. Identification was based on colony characters and morphology of sporulating structures under a compound microscope (Mathur and Kongsdal, 2003). The experiment was repeated three times.

4.2.5. Morphological identification

The morphological identification of the pathogen was done using the standard cellophane technique preparation. The *Alternaria* spp. isolated from the various seed lots were compared to the three-dimensional sporulation pattern of the *Alternaria* spp. described by Simmons (1990; 1993; 1994;1995). The nineteen isolates isolated from seedling blight lesions after germination and during the agar plate method were cultured on PDA for 14 days at 25°C under 12 hours alternating cycles of UV light and darkness. A piece of clear cellophane tape was gently pressed onto the surface of the mycelium, and gently lifted and placed onto a glass slide. The morphology of the *Alternaria* isolates was observed and examined under a Zeiss light microscope without the use of a cover slip.

4.2.6. Statistical analysis of data

The germination test, agar plate seed health test and component seed test data for the 13 sunflower seed lots was analysed using ANOVA after arcsin/angular transforming the percentage data. Separation of means was done using Fisher's least significant difference (LSD) test ($P \leq 0.05$) which was computed to study the interaction among the 13 seed lots. All experiments were repeated three times and the data was statistically analysed using the SAS Version 9.1 (Institute, Inc., 2005) statistical package. To analyse the correlation between germination, seedling blight, seed coat and cotyledon/embryo infection, and seed infection, the statistical package EViews Version 3.1 (Quantitative Micro Software, 2000) was used.

4.3. Results

4.3.1. Standard germination test

Thirteen sunflower seed lots were evaluated to assess their germination potential and health of emerged seedlings from the seeds. Seed germination ranged from 58 to 94% among the various seed lots (Table 4.1). Seed lots PAN 7531 (Bapsfontein) and PAN 7050 (Greytown) had germination percentages above 90%. The remainder of the seed lots had germination percentages above 80%, except for PAN 7063 (Cl) and Agsun 8251. However, there was no significant difference in germination percentage between the two Agsun 8251 seed lots from Ventersdorp

and Viljoenskroon. There was a significant difference in germination of seed lots of the same cultivars of PAN 7050 and PAN 7351 but from different provinces.

The cotyledons of some of the seedlings after the germination tests had seedling blight lesions. The lesions were mostly circular with a dark brown centre. No chlorotic halo encircling the lesions was evident (Fig. 4.1). The fungi isolated from the lesions were *Alternaria* spp. PAN 7531 (Bapsfontein) had the lowest seedling blight infection. PAN 7050 (Bapsfontein), PAN 7351 (Greytown) and Agsun s181 had the highest seedling blight of over 46% infection (Table 4.1).

Table 4.1: Germination percentages and percentages of sunflower (*Helianthus annuus* L.) seedlings affected with seedling blight caused by *Alternaria* spp. after germination tests.

Seed lot	**Region**	**%Germination**	**%Seedling blight**
PAN 7050	Bapsfontein	81 cd	60 a
PAN 7531	Bapsfontein	94 a	7 h
PAN 7057	Bapsfontein	85 bcd	23 cdefgg
PAN 7063 (Cl)	Bapsfontein	58 e	32 c
PAN 7049	Bapsfontein	86 bc	11 gh
PAN 7351	Greytown	86 bc	53 ab
PAN 7050	Greytown	91 ab	25 cdef
Agsun s661	Viljoenskroon	85 bcd	25 cdef
Agsun 5671	Bloemfontein	83 cd	13 efgh
Agsun s181	Viljoenskroon	81 cd	46 b
Agsun 8251	Ventersdorp	84 bcd	24 dce
Agsun 8251	Bloemfontein	79 bcd	16 efgh
NK ferti	Viljoenskroon	87 abc	29 c

*Means followed by the same letter in a column indicate no significant difference in respective tests, LSD=8, $P \leq 0.0001$.

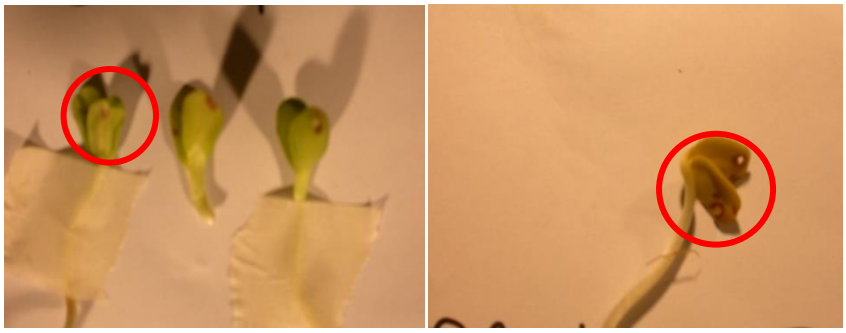

Figure 4.1: Lesions on sunflower (*Helianthus annuus* L.) cotyledons caused by *Alternaria* spp. during the germination test.

4.3.2. Agar plate seed health method

The main fungal species that were present on the sunflower seed lots using the agar plate method were *Alternaria* followed by a low occurrence of *Stemphylium, Rhizopus* and *Trichoderma* species (Table. 4.2). The fungal species that was mostly present on the sunflower seed lots using the agar plate method was from the genus *Alternaria* (Fig. 4.2). Agsun s181 seed lot had the most *Alternaria* infection with an infection percentage of 98%, whereas the Bapsfontein PAN 7531 seed lot was the least infected with the percentage infection of seed lot (Table 4.3).

Seed lots from Viljoenskroon had an infection percentage of over 90%, whereas seed lots tested from Bloemfontein had an average infection of 67% regardless of the seed lot or cultivar that was tested. In addition to the findings, Agsun 8251 seed lots from Ventersdorp and Bloemfontein had a significant difference in percentage infection (Table 4.3).

Table 4.2: Mycoflora associated with sunflower (*Helianthus annuus* L.) seeds in seed health testing of different sunflower seed lots collected from various parts of South African sunflower growing areas.

Seed lot	**Region**	**Percentage seed infection by fungal species of:**			
		Alternaria	***Stemphylium***	***Rhizopus***	***Trichoderma***
PAN 7050	Bapsfontein	84	-	-	-
PAN 7531	Bapsfontein	18	-	2	-
PAN 7057	Bapsfontein	74	3	-	-
PAN 7063 (Cl)	Bapsfontein	92	-	-	-
PAN 7049	Bapsfontein	52	-	-	-
PAN 7351	Greytown	56	-	5	-
PAN 7050	Greytown	36	-	13	-
Agsun s661	Viljoenskroon	95	-	-	-
Agsun 5671	Bloemfontein	69	-	-	11
Agsun s181	Viljoenskroon	98	-	-	-
Agsun 8251	Ventersdorp	95	1	-	-
Agsun 8251	Bloemfontein	67	6	-	1
NK ferti	Viljoenskroon	95	-	1	-

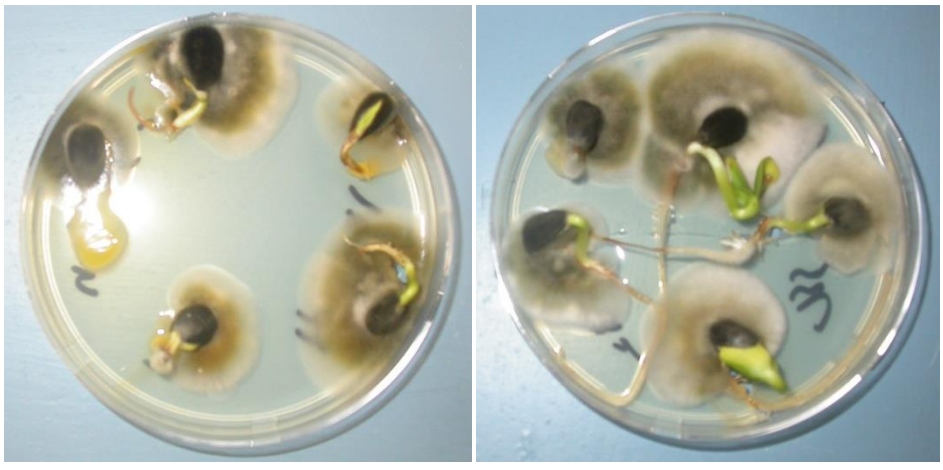

Figure 4.2: The colonization of sunflower (*Helianthus annuus* L.) seeds by *Alternaria* spp. using the agar plate method.

4.3.3. Seed component test using the agar plate method

Generally, seeds with a high seeds infection had high cotyledon and seed coat infection. However, seed component plating of the sunflower seed lots showed the cotyledon and embryo had more *Alternaria* infection than the seed coat (Table 4.3). NK ferti seed lot had the highest cotyledon infection of 94% and PAN 7531 had the lowest infection of 16%. Seed lots of NK ferti, PAN 7050 (Bapsfontein) and Agsun s181 had lesions prior to plating the cotyledon component on the agar, thus infection would have occurred preceding seed storage.

Table 4.3: Agar plate method and seed component test showing the percentage infection of sunflower (*Helianthus annuus* L.) seeds and seed parts caused by *Alternaria* species.

Seed lot	**Region**	**% infected seeds (Agar plate method)**	**Component % infection**	
			% cotyledon/embryo	**% seed coat**
PAN 7050	Bapsfontein	84 *cd	63 *def	44 *ef
PAN 7531	Bapsfontein	18 i	16 h	12 i
PAN 7057	Bapsfontein	74 de	62 ef	39 fg
PAN 7063 (Cl)	Bapsfontein	92 bc	75 c	60 ab
PAN 7049	Bapsfontein	52 gh	34 g	40 efg
PAN 7351	Greytown	56 fg	57 f	42 ef
PAN 7050	Greytown	36 hi	35 g	29 h
Agsun s661	Viljoenskroon	95 ab	84 b	59 bc
Agsun 5671	Bloemfontein	69 efg	70 cd	53 cd
Agsun s181	Viljoenskroon	98 a	63 def	34 gh
Agsun 8251	Ventersdorp	95 ab	63 def	44 ef
Agsun 8251	Bloemfontein	67 efg	62 def	43 ef
NK ferti	Viljoenskroon	95 ab	94 a	66 a

*Means followed by the same letter in a column indicate no significant difference in respective tests, LSD=8, $P \leq 0.0001$.

The correlation coefficient between seed germination and infected seed, seedling blight and infected seed parts was negative (Table 4.4). The correlation coefficient between germination and infected seeds and seed parts (embryo and cotyledon) ranged from -0.45 to -0.56.These results can be supported by Table 4.2 and Table 4.3, that in most cases when seed infection was high, the germination percentages of the seed lot would be lower, examples are seed lots of PAN

7050 (Bapsfontein) (81%), PAN 7063 (58%) and Agsun s181 (81%). On the contrary, seed lots of NK fertiand and Agsun s661 had a high percentage seed infection (both 95%), but still had high seed germination potential (87 and 85%, respectively). PAN 7531 (Bapsfontein) and PAN 7050 (Greytown) were the least infected seed lots (18 and 36% respectively), and had the highest germination potential (94 and 91%, respectively).The correlation coefficient between the infected cotyledon and embryo and infected seed coat was 0.9 (Table 4.4). The correlation coefficient between the infected cotyledon and embryo and infected seeds was 0.86.

Table 4.4: The correlation among germination, seedling blight and *Alternaria* infected sunflower (*Helianthus annuus* L.) seeds and seed components.

	Germination	Seedling blight	Agar plate	Infected C/E*	Infected seed coat
Germination	1.00	-0.24	-0.53	-0.45	-0.56
Seedling blight	-0.24	1.00	0.42	0.33	0.18
Agar plate	-0.53	0.42	1.00	0.86	0.75
Infected C/E*	-0.45	0.33	0.86	1.00	0.90
Infected seed coat	-0.56	0.18	0.75	0.90	1.00

*C/E is cotyledon and embryo.

4.3.4. Morphological identification

The nineteen *Alternaria* isolates that were isolated from the various seed lots were grouped into approximately three *Alternaria* species-groups. The morphological variation was observed in terms of colony pigmentation, conidial measurements and three dimensional sporulation patterns. Conidia and conidiophore morphological examination on PDA at 40x magnification of all nineteen isolates resulted in similar sporulation grouping as obtained by Simmons (1990; 1993; 1994; 1995). The *Alternaria* species that were isolated from the seeds were *A. helianthicola, A. alternata,* and *A. tenuissima*.

Group 1 was characterised in chapter 3 and was described to be *A. helianthicola* (PPRI 11433). Group 2 was characterized by short primary conidiophores and conidial chains that are seldom branched. The conidia formed had slender beaks. The conidiophores arose singly, simple, straight and were approximately 15 to 20 µm long. The conidia range from 12 to 30 µm in length

and were found to be the smallest closer to the conidiophores. The width of the conidia range from 8 to 12 µm. The colony growth pattern was circular with concentric circles within the colonies due to the alternating 12 hours light and darkness regimes (Fig. 4.3). The colony pigmentation was pale greyish-green and the texture was woolly. This group has the description of *A. tenuissima.*

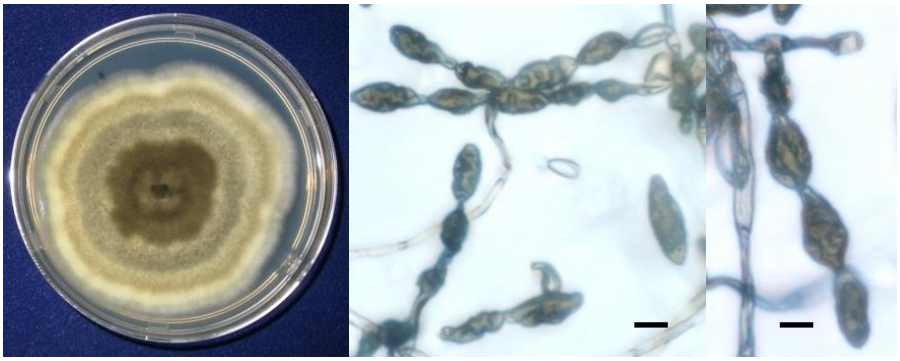

Figure 4.3: Culture and sporulation structures of *Alternaria tenuissima* isolated from lesions of sunflower (*Helianthus annuus* L.) seedlings. Each bar represents 10 µm. Light microscope of 40x magnification was used.

Group 3 was characterized by short primary conidiophores and conidial chains that were branched out (Fig. 4.4). The conidia formed were obclavate, and were pale to olivaceous green, smooth, with up to 6 transverse septa. Conidiophores arose singly or in branches, simple and pale to mid olivaceous green to pale green, and grew up to 50µm long. The conidia ranged from 18 to 34 µm in length and the width of the conidia was 12 to 15µm. It was not classified as *A. arborescens* as it had less branching and shorter conidiophores than *A. arborescens*. The colony growth pattern was circular with concentric circles showing within the colony (Fig 4.4). The colony texture was woolly. This group was represented by the *Alternaria* isolate 18 culture which fits the description of *A. alternata*.

Figure 4.4: Culture and sporulation structures of *Alternaria alternata* isolated from lesions of sunflower (*Helianthus annuus* L.) seedlings. Each bar represents 10 µm. Light microscope of 40x magnification was used.

4.4.Discussion

The importance of seed quality tests is to ensure that the farmer uses the best quality seeds and that the farmer avoids spreading disease, as many plant pathogens are disseminated predominantly by seed (Neergaard, 1977). Detection of *Alternaria* spp. has been studied by Neergard (1977), Pryor *et al*. (2003) and Kim and Mathur (2006). However, detailed infection aspects of these fungi in sunflower seeds and their pathological effects on seed germination and seedling growth of sunflower have been little studied. This study reported on the results of germination tests of 13 sunflower seed lots followed by the agar plate method to determine the fungal species found in the seed lots.

The negative correlation between seed germination and infected seed, seedling blight and infected seed parts implies that germination is inversely proportional to seed infection and seedling blight. Therefore, germination will be decreased or hampered with increase of seed infection, whether it is the seed coat, embryo or cotyledon that is infected. The correlation coefficient between germination and seed infection was -0.56. The negative correlation implies that seeds infected with *Alternaria* species have a 56% chance of poor germination. The correlation coefficient of -0.56 is not regarded as a strong correlation. There are several factors that may play a role to weak correlation. Firstly, *Alternaria* is a cosmopolitan fungal genus that

includes saprophytic and pathogenic species (Peever *et al.,* 2004). Thus, some of the *Alternaria* species found on the seed during the agar plate test may have been merely saprophytic species. Secondly, some *Alternaria* species may cause quiescent infection until the environmental conditions are conducive for disease (Rotem, 1994). Finally, some *Alternaria* species only cause contamination of the seed coats but do not cause disease (Neergaard, 1977). Therefore, the species that were isolated from the infected seeds may be weak parasites, because they did not lower the germination percentage but were able to cause seedling blight lesions on cotyledons.

Consequently, the weak correlation between seed infection and germination was evident in this study since some seed lots such as NK ferti and Agsun s661 which had a high infection percentage but still had a high germination percentage had a high percentage seed infection. This did not concur with the results of Coles and Wick (2001) who stated that seed samples with a high incidence of *Alternaria* species infestation generally have lower levels of germination compared to those low levels of infestation .Studies on the correlation of carrot (*Daucus carota* L.) seed germination and *A. radicina* seed carrot seed infection, by Kim and Mathur (2006), also showed that seed germination was lower for the seed lots that had the highest seed infection and these seeds had a significantly higher seedling blight infection. The correlation coefficient between the infected cotyledon and embryo and infected seed coat (0.9) indicates a high chance for the infected seed coat to contaminate the inner seed parts should the seed coat be infected. The correlation coefficient between the infected cotyledon and embryo and infected seeds (0.86) indicates a higher chance to isolate the pathogen from the inner parts of the seeds if the seeds are infected.

The agar plate method test showed that *Alternaria* species were the most common fungal species found on the sunflower seeds as compared to the other seed-borne fungi. The incidence of the *Alternaria* species infection in the seed lots tested ranged from 18 to 98%. Sunflower is normally sown in the summer and during this period the environmental conditions favour the proliferation of *Alternaria* species (Reis *et al.*, 2006). Over the past five years during the sunflower growing season in Ventersdorp, Viljoenskroon and Pretoria, the average temperature and the average relative humidity has been 30°C and 92%, respectively (Agrometeorology Staff, 2012). In this study, seed infection in these areas was seen to be above 90%. Bloemfontein has on the other

hand had an average temperature and relative humidity of 25°C and 80% (Agrometeorology Staff, 2012). Seed infection by *Alternaria* species on sunflower seed lots grown in Bloemfontein was less than 70%. Epidemics of Alternaria blight of sunflowers are most common and severe in areas that experience extended periods of wet weather in the summer accompanied by mean and daily temperatures between 25 and 30°C (Allen *et al.*,1982).

Seed-borne infections by *Alternaria* species may be both internal and superficial because infection of sunflower seeds by *Alternaria* species takes place during the advance stage of seed development, thus the seeds may be affected through their flowers or the seed coat (Rotem, 1994). This statement supports this study because the *Alternaria* pathogen was found both on the seed coat and the cotyledon during the seedling component test. *Alternaria* was isolated more often from the cotyledons/embryo than the seed coat. High levels of internal infection indicate that the fungus was not a chance contaminant but its presence resulted in the direct attack of the seeds.During systemic infections, the fungus grows into the ovule and later develops from the seed further into the seedling (Neergaard, 1977). However, Szopinska *et al.* (2007) stated that the seed coat is the common site of an infection for most seed-transmitted Mitosporic fungi (formerly known as Fungi Imperfecti). The lower *Alternaria* seed coat infection percentage may have been caused by the effect of submerging the thin layered seed coat in the harsh sodium hypochlorite solution. Seed disinfection may have decreased the number of seeds infested with *Alternaria* fungi (Szopinskaand Bralewski, 2006). The seed coats may have been infected from the outside by conidia being disseminated by wind to young florets of healthy heads. In some cases the pathogen may be found contaminating the seed surface (Suryanarayana, 1978).

The identification of the *Alternaria* species isolated from the seeds using morphological techniques was based on the variations observed in terms of conidial measurements and three-dimensional catenulation. Colony pigmentation was not a valuable criterion because the isolates of *Alternaria* are genetically variable and any given mycelium may become heterokaryotic (Slavov *et al.,* 2004), instead conidial size range and the three dimensional sporulation patterns were mostly used as they are critical for differentiating similar species in the small-spored group (Simmons, 1990).

There were similarities in the nineteen isolates as compared to the small-spored *Alternaria* described by Simmons (1995). The general appearance of the small-spored *Alternaria* is that of relatively small conidia that may reach dimensions of about 50 x 18 µm. Small-spored conidia typically do not exceed 50 µm in culture. Many of the conidia had a short conical, narrowly tapered beak, some others were beakless, and still others had short apical secondary conidiophores (Simmons, 1995).

In conclusion, the incidence of *Alternaria* species shows that these fungi are common in all the sunflower producing areas of South Africa and they are not confined to any specific cultivar of sunflower as they were detected in all the seed lots of this study. Small-spored *Alternaria* species have the potential to affect sunflower seed germination and also cause seedling blight on the cotyledons. The high incidence of *Alternaria* species on sunflower seed lots is a cause for concern as planting infected seed poses a threat to the sunflower industry by causing early widespread epidemics.

Further studies are required to differentiate between saprophytic, quiescent and pathogenic species of *Alternaria* and what differentiates these species. The presence of the fungi on the embryo and cotyledon of the seeds suggests that fungicidal control may be achieved provided chemicals with penetration abilities or soak treatments can eliminate internal infections. However, most importantly the use of pathogen-free seeds will be an essential component of an integrated management of seed-borne *Alternaria* species on sunflower.

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Chapter 5: Molecular identification of *Alternaria* spp. isolated from sunflower (*Helianthus annuus* L.) in South Africa

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The genus *Alternaria* has over a hundred species that are ubiquitous and may either be plant pathogenic or saprophytic. *Alternaria helianthi* is the main cause of sunflower blight, but recently small-spored *Alternaria* have been found to cause severe leaf spots and blights. Morphological characterization of these small-spored *Alternaria* species has been found to be unreliable due the overlap in cultural characteristics between the various species. Molecular characterization using the rDNA ITS operon, β-tubulin gene and the EF-1α gene was done to support the morphological characterization of *Alternaria* spp. found on sunflower. The rDNA ITS operon showed extensive length polymorphism among the *Alternaria* species. The polymorphism observed did not allow proper identification of the isolates. The *Alternaria* pathogen on sunflower seeds was classified as *A. alternata* according to the β-tubulin gene and the EF-1α gene sequences. The isolate from sunflower leaves may possibly be *Alternaria helianthicola* as no sequences of *A. helianthicola* exist on GenBank and no comparisons could be made.

Keywords: sunflower, rDNA ITS, polymorphism, β-tubulin gene, EF-1α gene, *A. alternata*

5.1. Introduction

Alternaria helianthi (Hansf.) Tubaki and Nishihara is the main cause of Alternaria leaf spot of sunflowers [*Helianthus annuus* L.] (Allen *et al.,* 1983). Nine other *Alternaria* species have been reported on sunflower, including *A. alternata* (Fries) Kiessler, *A. zinnae* Ellis, *A. tenuissima* (Fries) Wiltshire, *A. leucanthemi* Nelen (syn *A. chrysanthemi* Simmons and Grosier), *A. helianthicola* Rao and Rajagopalan, *A. longissima* Deighton and MacGarvey, *A. helianthinficiens* Simmons, and *A. protenta* (Lapagodi and Thanassoulopoulos, 1998). *Alternaria* species are ubiquitous and are highly resistant to adverse weather, because they can develop under a wide range of temperatures and can utilize locally available sources of moisture (Green and Bailey, 2000).

The genus *Alternaria* includes nearly 100 species that occur worldwide (Rotem, 1994; Pryor and Gilbertson, 2000) that may either be plant pathogenic or saprophytic species (Konstantinova *et al.,* 2002). The characterization and taxonomy of *Alternaria* species has been based on morphology and sometimes host association (Simmons, 1990; Rotem, 1994). However, when the genus as a whole is considered, species identification becomes more difficult because some species have ranges of spore dimension that overlap those of other species (Pryor *et al.*, 2003). Many *Alternaria* species produce small spores aggregating in branching chains and collectively these species can be found in nearly all agricultural systems causing a variety of diseases (Agrios, 2005). Morphological differences in *Alternaria* species are subtle, but the variations in pathogenicity may be great (Pryor *et al.*, 2003).

Identification of small-spored *Alternaria* species has been tricky since small-spored *Alternaria* species have similarities in spore sizes, shape and catenation. Examples of such small-spored *Alternaria* include: *A. alternata, A. tenuissima, A. arborescens* E.G. Simmons and *A. infectoria* E.G. Simmons (Pryor *et al.,* 2003). Catenation and conidial morphology of *Alternaria* species is affected by the conditions of growth such as substrate, light and humidity and thus may be unreliable both for implying phylogenetic relationships and characterization of various species (Rotem, 1994). In addition, the Alternaria infectoria species-group is the only group in

Alternaria where some members have a teleomorph state, *Lewia* ME Barr and EG Simmons (Andersen *et al*., 2009).

Recently, molecular methods have been employed to classify or segregate *Alternaria* species, but with variable results (Andersen *et al.,* 2002). The molecular tools that have been used for *Alternaria* spp. include sequence analysis of rDNA such as the nuclear internal transcriber spacer (ITS) region (Konstantinova *et al.,* 2002; Pryor *et al.,*2008); protein coding genes such as the β-tubulin gene (Quayyum *et al.,* 2005), and the translation elongation factor (EF-1α) gene (Dagno *et al.,* 2011; Gat *et al.,* 2012). However, small-spored *Alternaria* species are still taxonomically a challenging group of fungi with few morphological or molecular characteristics that allow distinctive discrimination among the taxa (Andrew *et al*., 2009). The objective of this study was to identify *Alternaria* isolates recovered from sunflower leaves and seeds obtained from various sunflower growing areas of South Africa using molecular identification techniques.

5.2.Materials and Methods

5.2.1. Fungal isolates and seed lots

Twenty one *Alternaria* isolates were used in the present study. These isolates were obtained from leaf blight infected sunflower leaves and seeds of various seed lots received from sunflower farms and their identities were confirmed by morphological techniques. Samples of diseased sunflower leaves were collected during a field trip to northern KwaZulu-Natal (30°36’01” S-29°04’56.54”E) and Potchefstroom (26°44’09.95”S-27°04’24.83”E). Specimens were placed in individual paper bags to prevent rapid desiccation, and transported to the laboratory in an ice box. Approximately 5 mm^2 of the leaf was cut with a clean scalpel blade. The leaf pieces included both healthy and infected tissue. The leaf pieces were disinfected by immersion in a 1% sodium hypochlorite solution for 5 min and rinsed in sterile distilled water for a minute. The excised leaf pieces were placed on potato dextrose agar (PDA) containing chloramphenicol (0.01 g chloramphenicol was added to 1 litre PDA before pouring into plates). The Petri dishes were incubated at incubated at 25°C in a 12hour UV-light/12hour dark cycle for 3 days. The *Alternaria* cultureswere purified from the master plate and plated onto PDA media containing

the same antibiotics as above. The plates were incubated at 25°C in a 12hour UV-light/12hour dark cycle for 7 days. This light regime was followed to promote sporulation.

Further isolations were made from sunflower seeds of thirteen seed lots that were infected with *Alternaria* species during the standard germination tests and agar plate methods. The *Alternaria* spp. that were isolated from the seedling blight lesions during the standard germination tests and the agar plate method were placed on PDA containing chloramphenicol. The plates were incubated at 25°C in a 12hour UV-light/12hour dark cycle for 7 days. A single spore culture of each *Alternaria* isolate was prepared by pouring 4 ml of sterile distilled water on a 14 day-old culture. Two ml of the suspension was spread uniformly on potato dextrose agar (PDA) and incubated at 25°C for 24 hours. The plates were examined under the microscope to locate single, germinated spores, which were re-cultured on fresh PDA and incubated at 25°C in a 12hour UV-light/12hour dark cycle for 7 days.

5.2.2. DNA extraction, PCR amplification and sequencing

A total number of 21 fungal isolates were identified morphologically. From the 21 isolates, five isolates were identified by using three gene regions of ITS rDNA operon, β-tubulin and EF-1 genes. Fungal mycelium and conidia from the various isolates grown on PDA for 2 weeks at 25°C were scraped into eppendorf tubes. DNA extraction was done using a Zymo DNA extraction kit (Zymo Research, California). Amplifications were performed using three sets of primers (Table 5.1): 1) ITS-1F and ITS-4 (McKay *et al*., 1999; Quayyum *et al.,* 2005); this primer pair was used for the PCR amplification of rDNA containing the internal transcribed spacer (ITS) 1, the 5.8S gene and ITS2; 2) β-tubulin gene, β-tubulin 1a and β-tubulin 1b (McKay *et al*., 1999; Quayyum *et al.,* 2005; Park *et al*., 2008; Gat *et al.,* 2012); 3) Translation elongation factor (EF-1α) gene, EF1α- 728F and EF1α -986R (Park *et al*., 2008; Gat *et al.,* 2012). A 25µl reaction volume containing a reaction mixture of 18.25 µl of sterile double-deionised water, 5U My Taq buffer, 0.25U Taq DNA polymerase, 0.25 µl of the respective primer sets (200 nM) and 1 µl template DNA (15 ng/µl) was used.

Table 5.1: Primers used in the amplification of the DNA sequences of internal transcribed spacer (ITS), β-tubulin and EF-1 genes used in the present study

Primer name	Nucleotide sequence (5'-3')	Expected fragment size (bp)
ITS-1F	TCCGTAGGTGAACCTGCG[a]	~ 630
ITS-4	TCCTCCGCTTATGTATATGC[a]	~ 630
β-tubulin 1a	CAGCTCGAGCGTATGAACGTCT[b]	~ 480
β-tubulin 1b	TGTACCAATGCAAGAAAGCCTT[b]	~ 480
EF-1α 728 F	CATCGAGAAGTTCGAGAAG[c]	~ 260
EF-1α 986 R	TACTTGAAGGAACCCTTAC[c]	~ 260

[a,b]McKay *et al*., 1999; [a,b]Quayyum *et al.,* 2005; [b,c]Park *et al*., 2008; [b,c]Gat *et al.,* 2012

PCR amplifications were performed in an MJ Mini: Personal Thermal Recycler (Bio-Rad). For the ITS primers, the program consisted of an initial step of 3 min at 94°C, followed by 35 cycles of denaturation at 94°C for 30 sec, and elongation at 72°C for 1 min. A final extension was performed at 72°C for 10 min. For the β-tubulin and EF-1α primers, the program consisted of an initial step of 5 min at 94°C, followed by 35 cycles of denaturation at 94°C for 45 sec, and elongation at 72°C for 1 min. A final extension was performed at 72°C for 10 min. PCR products were analysed in 1.5% agarose gels, stained with ethidium bromide and visualized under UV light. An hpII ladder was used as a molecular weight marker.

PCR products were purified with the GeneJet™ PCR purification kit (Inqaba Biotec, Pretoria, South Africa). Post clean-up of the purified PCR products was done by adding 10 µl of sterilized Sabex water, 2 µl of 3M sodium acetate and 50 µl of absolute ethanol. The mixture was incubated in ice for 10 min and centrifuged for 30 min at 13 000 rpm using a Hermile (Lasec SA (Pty) Ltd) centrifuge. The supernatant was discarded and 250 µl of 70% ethanol was added to the purified PCR products and centrifuged for 5 min at 13 000 rpmat 4°C. The purified PCR products were sequenced in both directions using an ABI Prism DNA Automated Sequencer (Perkin Elmer). The primers used for sequencing were the same as those used to direct the amplification of the given DNA fragment. The sequences of both the forward and the reverse strand of each fragment were determined from the sequencing.

Sequences of the forward and reverse runs were combined to form the consensus sequences. Consensus sequences of the *Alternaria* isolates that were successfully sequenced were aligned

using the BioEdit Sequence Alignment v 7.0.0 (Hall, 1999).BioEdit Sequence Alignmentallowed for manual adjustments of sequence alignments. The sequences were trimmed on the 5'- and 3'-ends to aid alignment. The sequences were compared with the sequences of related species found in GenBank (Table 5.2) (http://www.ncbi.nlm.nih.gov/blast/) by alignment using a computer program (online MAFFT). Phylogenetic analyses were performed using MEGA v 5.0 (Tamara *et al.*, 2011). *Stemphylium vesicarium* (teleomorph *Pleospora herbarum*) was rooted as an outgroup in all three analyses. For each set of DNA sequences, phylogenetic trees were constructed using distance methods. The neighbour joining tree was constructed using the number of differences method. For each analysis, 1000 bootstrap replicates were performed to assess the support for each clade.

Table 5.2: GenBank Accession numbers for *Alternaria* isolates used for phylogenetic analysis.

Description	Species	GenBank Accession number
TS (1F and 4)	*A. alternata*	GU566303.1
ITS (1F and 4)	*A. alternata*	JQ320281.1
ITS (1F and 4)	*A. alternata*	AB693900.1
ITS (1F and 4)	*A. arborescens*	HQ443201.1
ITS (1F and 4)	*A. arborescens*	JQ406953.1
ITS (1F and 4)	*A. brassicicola*	JF710519.1
ITS (1F and 4)	*A. citri*	AY154705.1
ITS (1F and 4)	*A. gaisen*	AF314581.1
ITS (1F and 4)	*A. helianthi*	JF10539.1
ITS (1F and 4)	*A. helianthi*	HM449992.1
ITS (1F and 4)	*A. helianthi*	JN208925.1
ITS (1F and 4)	*A. helianthi*	DQ156343.1
ITS (1F and 4)	*A. helianthi*	JF10540.1
ITS (1F and 4)	*A. helianthi*	HM489876.1
ITS (1F and 4)	*A. helianthi*	HM489877.1
ITS (1F and 4)	*A. infectoria*	AJ549824.1
ITS (1F and 4)	*A. longipes*	HQ343434.1
ITS (1F and 4)	*A. mali*	AF314575.1
ITS (1F and 4)	*A. porri*	JF422730.1
ITS (1F and 4)	*A. porri*	JF422727.1
ITS (1F and 4)	*A. solani*	JF796055.1
ITS (1F and 4)	*A. tenuissima*	JF710535.1
ITS (1F and 4)	*Stemphylium vesicarium*	JX424811.1
β-tubulin gene	*A. alternata*	GQ240308.1
β-tubulin gene	*A. alternata*	JN247835.1
β-tubulin gene	*A. alternata*	EU139350.1

β-tubulin gene	*A. arborescens*	AF397268.1
β-tubulin gene	*A. brassicicola*	JF417706.1
β-tubulin gene	*A. infectoria*	Y17083.1
β-tubulin gene	*A. porri*	JF3315861.1
β-tubulin gene	*A. radicina*	EU139378.1
β-tubulin gene	*A. smymii*	EU139351.1
β-tubulin gene	*A. solani*	JF417707.1
β-tubulin gene	*A. tenuissima*	AF397256.1
β-tubulin gene	*A. tenuissima*	AF397265.1
β-tubulin gene	*A. tenuissima*	AF397249.1
β-tubulin gene	*Lewia infectoria*	AM051271.1
β-tubulin gene	*L. infectoria*	AF397263.1
β-tubulin gene	*S. vesicarium*	JQ671944.1
EF-1α gene	*Alternaria alternata*	AY438647.1
EF-1α gene	*A. alternata*	EU139348.1
EF-1α gene	*A. molarum*	FJ214905.1
EF-1α gene	*A. radicina*	EU139417.1
EF-1α gene	*A. radicina*	EU139408.1
EF-1α gene	*A. radicina*	EU139414.1
EF-1α gene	*Embellisia abundans*	FJ214947.1
EF-1α gene	*L. infectoria*	FJ214922.1
EF-1α gene	*L. infectoria*	FJ214913.1
EF-1α gene	*L. infectoria*	FJ214934.1
EF-1α gene	*L. infectoria*	FJ214911.1
EF-1α gene	*L. infectoria*	FJ214912.1
EF-1α gene	*L. infectoria*	FJ214949.1
EF-1α gene	*Stemphylium vesicarium*	JQ672392.1

5.3. Results

5.3.1. DNA extraction, PCR amplification and sequencing

PCR products of ITS1F and ITS4, β-tubulin and EF-1α sequences were successfully amplified with approximate sizes of ~600bp, ~480bp and ~250bp, respectively. These sequences were aligned with known ITS rDNA, β-tubulin and EF-1α nucleotide sequences of *Alternaria* species from GenBank database. The GenBank accession numbers (http://www.ncbi.nlm.nih.gov/blast/) for all ITS rDNA, β-tubulin; and EF-1α nucleotide sequences used in this study are stipulated in Table 5.2.

5.3.2. ITS operon phylogenetic analysis

Amplifications carried out on DNA from five *Alternaria* isolates targeting ITS1F and ITS4 regions produced a reproducible PCR product. The size of the sequences ranged from 540 to 600 base pairs (Fig. 5.1). The neighbour-joining based on the two rDNA regions revealed extensive polymorphism between the studied *Alternaria* species. *Alternaria alternata, A. arborescens, A. tenuissima, A. citri, A. gaisen, A. mali, A. longipe*s, *A. brassicicola* were clustered together and supported by a bootstrap value of 100%. *A. solani* and *A. porri* which are large-spored were highly similar to other small-spored *Alternaria* species. This was supported by a bootstrap of 100% (Fig. 5.2). However, the large spored *A. helianthi* grouped differently from small-spored *Alternaria* species including *A. infectoria. A. infectoria* was a sister taxa with the small-spored *Alternaria* supported by a bootstrap value of 99%. Therefore, the ITS rDNA did not resolve the genus *Alternaria* to species level.

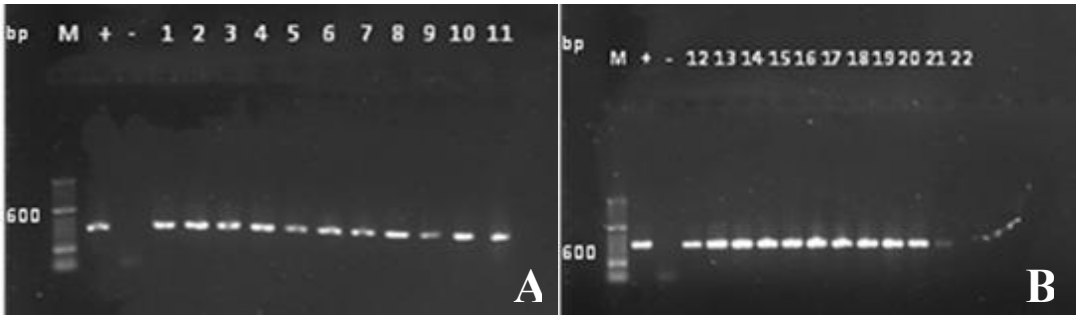

Figure 5.1: Gel electrophoresis of PCR products with A) *Alternariaalternata* specific primers, (M) molecular weight marker (1kb ladder), (-) is the negative and (+) is the positive control; lanes 1-11: *Alternaria* spp. isolated from sunflower (*Helianthus annuus*)seeds 2: B) ITS 1-F/ITS4 primers, (M) molecular weight, (-) is the negative and (+) is the positive control; lanes 12-22;*Alternaria*spp. isolated from sunflower seeds.

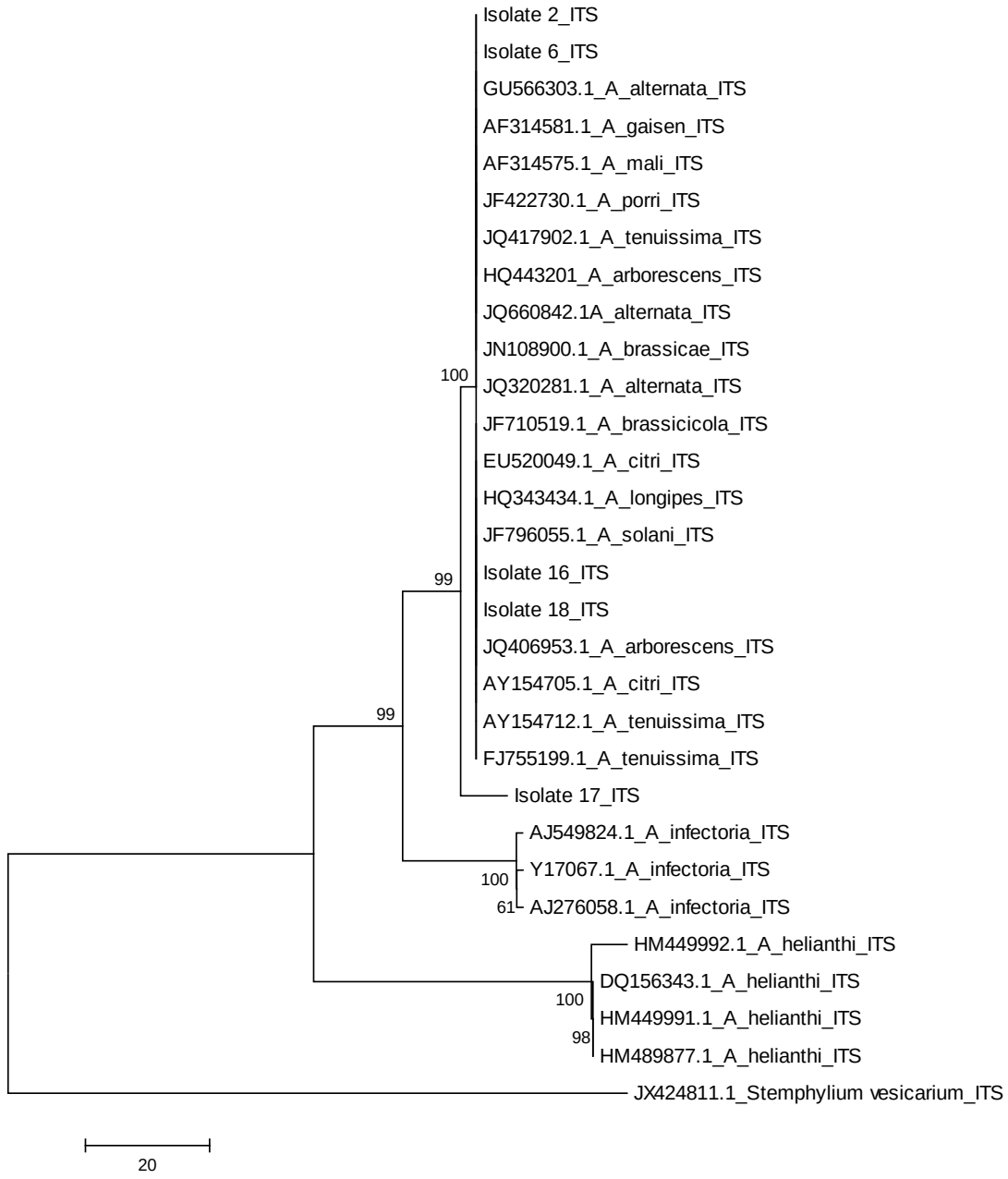

Figure 5.2:Neighbour-joining tree of *Alternaria* species sampled from sunflower (*Helianthus annuus*)leaves and seeds. The tree was generated by MEGA v 5.05 using the Number of Differences method to estimate the phylogeny of *Alternaria*species for sequence data fromITS1F and ITS4combined regions of the ribosomal genes. Values associated with branches indicate the degree of bootstrap support expressed as percentage of 1 000 bootstrapped trees in which corresponding clades are present. *Stemphylium vesicarium* was rooted as the outgroup in the analysis.

5.3.3. β-tubulin gene phylogenetic analysis

Amplification of the β-tubulin gene yielded amplicons that varied in size from 440 to 470bp. Phylogenetic analysis of the β-tubulin gene revealed more resolution between *Alternaria* species than the ITS region (Fig. 5.3). The *Alternaria* isolates from sunflower seeds clustered with *A. alternata* reference sequences. The bootstrap values for *A. alternata* and *A. brassicicola* were very low (21%) and thus these two species were indistinguishable based on the β-tubulin gene, compared to the rDNA ITS operon. *A. arborescens* and *A. tenuissima* clustered together with *A. infectoria* with a bootstrap support of 99%. *A. solani* and *A. porri* were distinct from the small-spored *Alternaria* species, which was the expected result.

5.3.4. Translation elongation factor phylogenetic analysis

Analysis of the EF-1α gene phylogeny showed four well-supported clades. The four clades were supported by bootstrap values of ≥92%. Clade 1 contained fungal isolates that were highly similar to *A. alternata* and was supported by a bootstrap value of 91%. Isolate 2 was distinct from the other *Alternaria* isolates in Clade 1. Clade 2 contained *A. radicina* as the sister taxa to the Alternata species-group supported by a bootstrap value of 59%. Clade 3 was *A. malorum* and *Embellisia abanduns*. *E. abanduns* showed 99% similarity to the *Alternaria* species during the NCBI blast. Clade 4 was *A. infectoria* and its teleomorph state *Lewia infectoria*. Clade 4 and clade 3 were a monophyletic group supported by a bootstrap of 96% (Fig. 5.4). No elongation factor sequences of the species of *A. arborescens, A. tenuissima, A. brassicicola* or *A. solani* were found in GenBank. Thus, these species could not be used for comparing with the *Alternaria* isolates in this study.

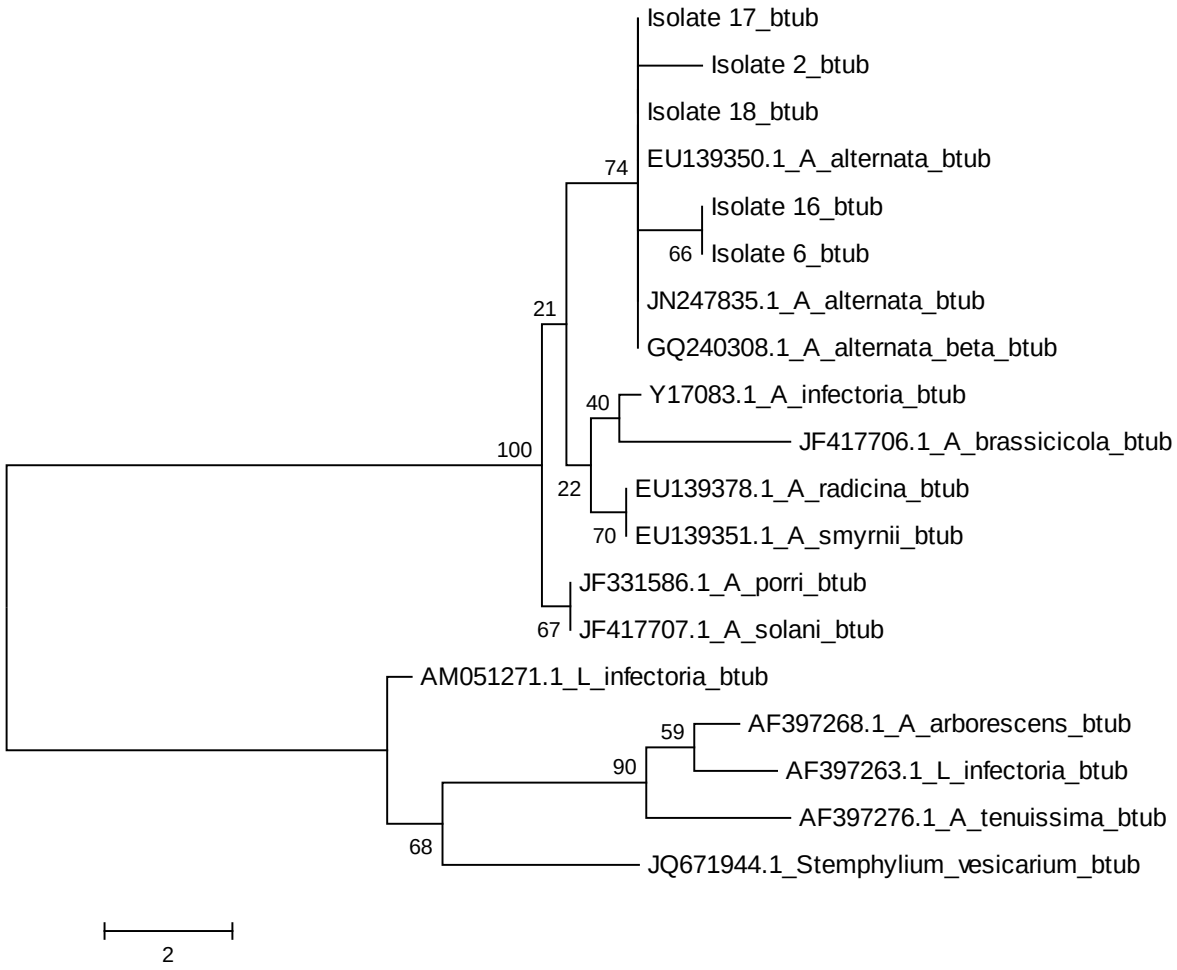

Figure 5.3: Neighbour-joining tree of *Alternaria* species sampled from sunflower (*Helianthus annuus*)leaves and seeds. The tree was generated by MEGA v 5.05 using the Number of Differences method to estimate the phylogeny of *Alternaria* species for sequence data from β-tubulin 1a and β-tubulin 1b combined regions of β-tubulin gene. Values associated with branches indicate the degree of bootstrap support expressed as percentage of 1 000 bootstrapped trees in which corresponding clades are present. *Stemphylium vesicarium* was rooted as the outgroup in the analysis.

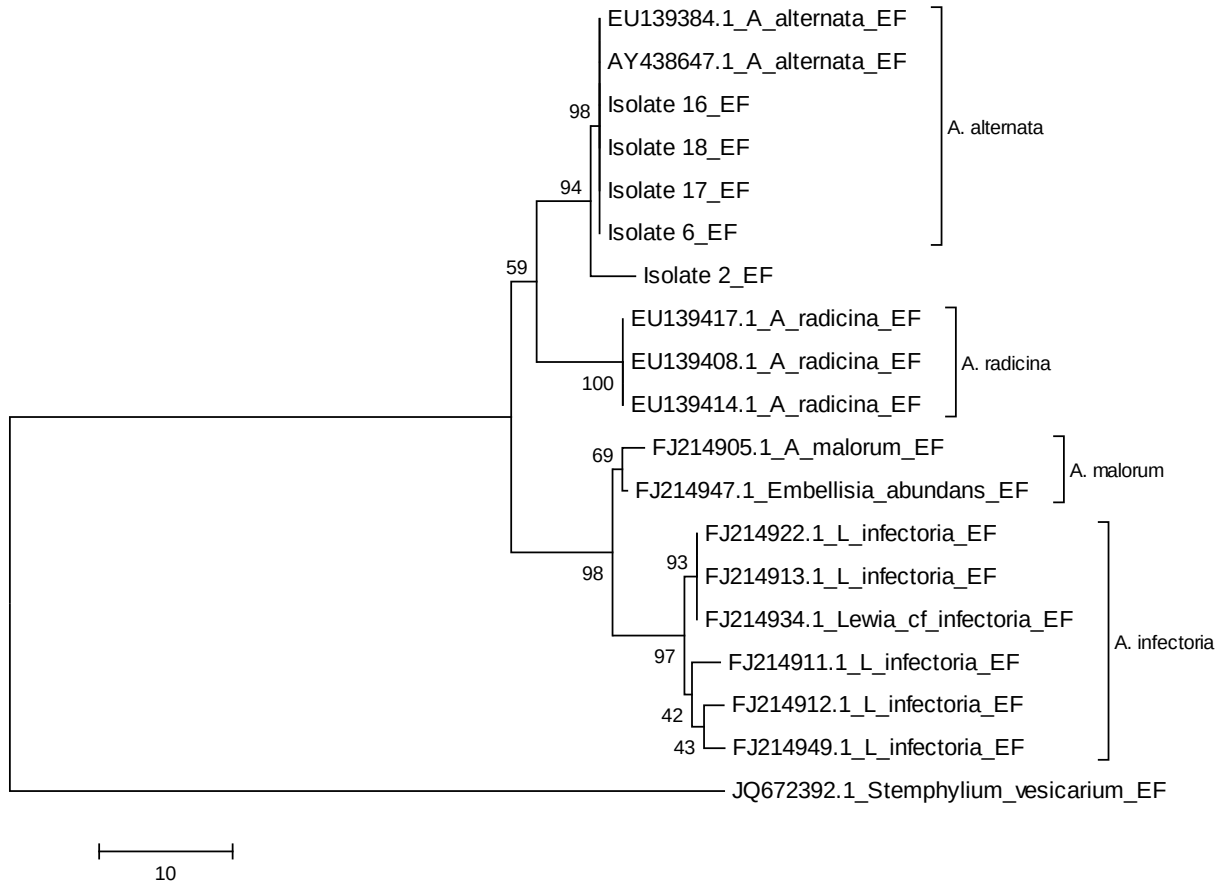

Figure 5.4: Neighbour-joining tree of *Alternaria* species sampled from sunflower (*Helianthus annuus* L.) leaves and seeds. The tree was generated by MEGA v 5.05 using the Number of Differences method to estimate the phylogeny of *Alternaria*species for sequence data from EF-1α (EF728 and EF927) combined regions of the translation elongation factor gene. Values associated with branches indicate the degree of bootstrap support expressed as percentage of 1 000 bootstrapped trees in which corresponding clades are present. *Stemphylium vesicarium* was rooted as the outgroup in the analysis.

Table 5.3: A summary of the morphological and molecular identification of small-spored *Alternaria* species on sunflower (*Helianthus annuus* L.) collected from various sunflower producing/growing areas of South Africa

Isolate	**Source**	**Plant tissue**	**Morphological ID**	**ITS operon**	**β-tubulin gene**	**EF-1α gene**
1	Potchefstroom	Leaves	*A. alternata*	-	-	-
2	Greytown	Leaves	*A. helianthicola* (PPRI 11433)	*Alternaria* sp.	*A. alternata*	Inconclusive
3	Pretoria	Seeds	*A. tenuissima*	*Alternaria* sp.	-	-
4	Pretoria	Seeds	*A. helianthicola*	*Alternaria* sp.	-	-
5	Pretoria	Seeds	*A. tenuissima*	*Alternaria* sp.	-	-
6	Pretoria	Seeds	*A. tenuissima*	*Alternaria* sp.	*A. alternata*	*A. alternata*
7	Pretoria	Seeds	*A. helianthicola*	-	-	-
8	Pretoria	Seeds	*A. alternata*	-	-	-
9	Greytown	Seeds	*A. alternata*	-	-	-
10	Greytown	Seeds	*A. alternata*	-	-	-
11	Greytown	Seeds	*A. tenuissima*	-	-	-
12	Viljoenskroon	Seeds	*A. tenuissima*	*Alternaria* sp.	-	-
13	Viljoenskroon	Seeds	*A. alternata*	-	-	-
14	Bloemfontein	Seeds	*A. alternata*	-	-	-
15	Bloemfontein	Seeds	*A. tenuissima*	*A. alternata*	-	-
16	Viljoenskroon	Seeds	*A. alternata*	*A. tenuissima*	*A. alternata*	*A. alternata*
17	Ventersdorp	Seeds	*A. tenuissima*	*Alternaria* sp.	*A. alternata*	*A. alternata*
18	Bloemfontein	Seeds	*A. alternata*	*Alternaria* sp.	*A. alternata*	*A. alternata*
19	Bloemfontein	Seeds	*A. alternata*	-	-	-
20	Viljoenskroon	Seeds	*A. alternata*	-	-	-
21	Viljoenskroon	Seeds	*A. tenuissima*	-	-	-

Based on the morphological identification, most of the *Alternaria* isolates were of the Alternataspecies-group. Two other groups included *A. tenuissima* and *A. helianthicola* of the Infectoria species-group. On the other hand, only two groups were identified using molecular techniques (Table 5.3). The Infectoria species-group downloaded from GenBank was distantly related from all the other small-spored *Alternaria* species according to all the gene regions used in this study. There was no consensus between the morphological identification and molecular identification (Table 5.3).

Using the ITS operon *Alternaria* isolates were identified an as *Alternaria* sp. because these small-spored species showed a high degree of similarity with a number of *Alternaria* species such as *A. alternata, A. tenuissima, A. brassicae, A. brassicicola, A. mali, A. gaisen, A. citri, A. longipes, A. solani* and *A. porri* (Fig. 5.2). The *A. helianthicola* sequences using rDNA ITS

region, β-tubulin gene and EF-1α gene have not yet been sequenced and therefore these sequences were not available on the NCBI GenBank. For that reason, the identity of isolate 2 was recorded as inconclusive using EF-1α gene because it does not form part of the *Alternata* clade (Fig. 5.4) and it was slightly different from the other isolates using the β-tubulin gene (Fig. 5.3).

5.4. Discussion

Pathogen and disease diagnosis are fundamental to virtually all aspects that relate to plant pathology (Ma and Michailides, 2007). The identification of *Alternaria* species has been based on morphology and sometimes host association (Rotem, 1994). Unfortunately, due to the ubiquitous nature of *Alternaria* species, they are found to grow almost everywhere, meaning they have the potential to grow on a non-host species. The sexual stage of most *Alternaria* spp. is unknown (Kusabe and Tsuge, 1995; Andersen *et al.,* 2002). Therefore, many *Alternaria* species exist in the vegetative phase, reproducing asexually, and this would display a low phenotypic variation (Guo *et al.,* 2004). The identification of *Alternaria* species still poses considerable difficulties owing to their similarities and polymorphism occurring even in pure cultures (Peever *et al.*, 2005).

In this study isolates were initially identified as *Alternaria* spp. based on morphology. Thereafter five of the isolates were further identified with molecular phylogenetic analyses of the ITS1F and ITS 4, β-tubulin; and EF sequences. The ITS region has gained widespread use in the taxonomy of many pathogenic species because it is a region that is highly conserved intra-specifically but variable between different genera and species (Buchan *et al.,* 2002). The ITS region showed high similarities among the various *Alternaria* species such as *A. alternata, A. brassicae, A. mali, A. gaisen, A. citri, A. lini, A. brassicicola, A. tenuissima, A. arborescens* and *A. longipes* (Fig. 5.2). These results concur with those of Pryor and Michailides (2001), who reported that the ITS region failed to resolve small-spored *Alternaria* species as phylogenetically distinct from *A. alternata* due to the minimal variation in the nucleotide sequences.

Conidia produced by fungi in the Alternata-species-group are 20-50µm long, while conidia produced by the Porri and Helianthi species-groups are generally more than 100µm long and are therefore considered as large-spored *Alternaria* species (Simmons, 1990; 1995). *Alternaria porri* and *A. solani* are part of the Porri species-group which includes species with large, long beaked and non-catenate conidia (Pryor and Gilbertson, 2000), and are therefore not morphologically similar to the small-spored *Alternaria* species which are small, seldom beaked and catenate. However, in this study the ITS operon was unable to segregate between certain large-spored and small-spored *Alternaria* species. Previous studies have shown that ITS operon sequencing analysis does not seem to resolve closely related species of *Alternaria* (Andersen *et al.*, 2002).

Kusaba and Tsuge (1995) and McKay *et al.* (1999) suggested small-spored *Alternaria* should not be separated at species level but should be classified as *A. alternata* with differentiation at species level as *formae specialis*. However, this suggestion was posed before β-tubulin and EF-1α genes were studied. This study showed that the β-tubulin gene and the EF-1α gene are able to segregate or resolve the dilemma between the small-spored *Alternaria* species. The β-tubulin gene was able to segregate the large-spored *Alternaria* species to the small-spored *Alternaria* species. *Alternaria solani* and *A. porri* of the Porri group were clustered together, although the bootstrap value was fairly low at 67%, this finding concurred with that of Pryor and Gilbertson (2000). *Alternaria smyrnii* and *A. radicina* formed part of the Radicina species group, in this study supported by 70% bootstrap and this corresponded with the results of Pryor and Bigelow (2003).The drawback of using the β-tubulin gene in this study was that it did not give a clear indication of the grouping of certain species. *A. infectoria* was seen to have clustered in the Alternata group, as well with *A. tenuissima* and *A. arborescens*. This may be due to the misidentification of the isolate using morphological techniques.

The EF-1α gene was able to segregate the various clades clearly. Most of the isolates clustered in the Alternata group except for isolate 2. *Embellisia abundans* and *A. malorum* were clustered together, supported by 69% bootstrap and concurs with Andersen *et al*. (2009). *A. malorum* and *E. abundans* formed as a sister taxa to the Infectoria species group, supported by a bootstrap of 98%. *Alternaria malorum* belongs to the Infectoria group (Andersen *et al*., 2009). The drawback

using the EF-1α gene was there were no sequences of *A. helianthicola, A. tenuissima* and *A. arborescens* on GenBank, thus no comparisons could be done using these sequences.

Isolate 2 clustered with the small-spored *Alternaria* species in the ITS operon and β-tubulin gene analysis, however, it was seen to be a different lineage within the respective clades. In the EF-1α gene phylogenetic analysis, isolate 2 formed as a separate lineage from *A. alternata* and the other isolates in the study. This may be attributed to the fact that isolate 2 was identified as *Alternaria helianthicola* of the Alternaria infectoria species-group based on the morphological identification. Other reasons that may attribute to the difference of the isolate to the Alternaria species-group may be either because of the host of the pathogenic fungus or to geographical variance (Peever *et al.*, 1999; Douhan *et al*., 2008; Hoeksema *et al.*, 2009).

The objective of this study was to identify *Alternaria* isolates recovered from sunflower leaves and seeds obtained from various sunflower growing areas of South Africa by molecular identification techniques compared to the morphological techniques. There was no consensus between morphological and molecular identification using ITS operon; β-tubulin gene; and EF-1α gene. This may have been due to misidentified samples as there are inconsistencies in identifying *Alternaria* species due to the phenotypic plasticity and different morphotaxanomic schemes between laboratories, thus causing more discrepancies in identification (Andrew *et al*., 2009). Therefore, it is crucial that accurate morphological identification is done, and this requires standardised methods and growth conditions, such as inoculation on nutrient rich media and incubation in the dark which promote the production of solitary conidia. Caution should be taken during morphological identification by doing thorough and standardised morphological examinations to identify *Alternaria* cultures, as pigmentation and conidial shape and size may be affected by incubation temperatures and type of media (Andersen *et al.*, 2002). Molecular identification is said to be the most accurate form of fungal species identification, but misidentification of isolates morphologically can cause more confusion in the taxonomy world as morphological identification precedes molecular characterization. In *Alternaria* species taxonomy, the ITS region is recommended only for preliminary identification to genus level as it cannot resolve or segregate small-spored *Alternaria* species. EF-1α gene was able to perfectly distinguish between the various *Alternaria* species, but more sequences need to be submitted to

the GenBank database for better comparisons. The *Alternaria* species isolated from the sunflower seeds were predominantly distinguished as *A. alternata* using the β-tubulin gene and EF-1α gene .The isolate from sunflower leaves may possibly be identified as *Alternaria helianthicola* as no sequences of *A. helianthicola* exist on GenBank and no comparisons could be made.

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Chapter 6: The influence of environmental conditions on disease severity of Alternaria leaf spot on sunflower (*Helianthus annuus*) caused by *Alternaria helianthicola*

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The causal organism of leaf spot on sunflower (*Helianthus annuus* L.) has been identified as *Alternaria helianthicola*. This study investigated the effect of temperature and relative humidity (RH) on lesion development. The *in vitro* test showed that the fungus has an optimum growth temperature of 25°C and maximum temperature of 35°C. Light was observed to promote hyphal growth and thus increased the radial growth rate of the fungus. Infective conidia were sprayed until run-off on 5- to 6-weeks old sunflower plants. Disease symptoms were seen as lesions surrounded by a chlorotic halo. For *in vivo* tests, approximately 12 hours of continuous RH is required for infection to progress provided the temperatures are optimal. Temperature had a significant effect on infection, with lesion development and enlargement observed to increase from 20 to 30°C, declining at 35°C.

Keywords: *Alternaria helianthicola, in vivo, in vitro,* temperature, humidity.

6.1. Introduction

The genus *Alternaria* is ubiquitous and highly resistant to adverse weather, because they can develop under a wide range of temperatures and can utilize locally available sources of moisture (Green and Bailey, 2000). Small-spored *Alternaria* species may develop survival strategies in their ecological niches by either being cosmopolitan saprophytes or opportunistic plant pathogens (Chou and Wu, 2002).

Pathogenic *Alternaria* species cause leaf spot on sunflower (*Helianthus annuus* L.), which is characterized by irregular dark, necrotic lesions with a greyish brown centre that is surrounded by a chlorotic halo on the leaves, stem, petioles and capitulum (Morris *et al*. 1983, Prathuangwong *et al.,* 1991). In severe infections, lesions become larger by coalescing, leading to blight, defoliation and death of plants(Mirza *et al.*, 1984, Cho and Yu, 2000). *Alternaria* leaf spot causes a decline in the photosynthetic area and also premature defoliation which adversely affects the growth and yield of the plant (Ojiambo*et al.,* 1998; Calvet *et al.,* 2005).

Alternaria species require high temperature and relative humidity to cause severe leaf spot disease (Rotem, 1994). Of the two parameters, temperature is the important because 4 to 12 hours of leaf wetness is required for infection to occur, provided the temperatures are optimal (Sangeetha and Siddaramaiah, 2007). The optimum temperature for *Alternaria* species germination and infection ranges from 24 to 27°C, and the maximum temperature is around 35°C (Abbas *et al*., 1995; Timmer *et al.,* 2003; Prasad *et al.,* 2008). The temperature range for sporulation is narrower than that of vegetative growth. Most *Alternaria* species sporulate optimally at 25°C, with 5 and 35°C as their minimum and maximum temperatures, respectively (Rotem, 1994).

Thus, *Alternaria* spp. are more virulent in tropical and subtropical regions since the combination of high temperatures (25-30°C) and relative humidity over 70% facilitates disease development (Allen *et al.* 1982; Green and Bailey, 2000; Reis *et al.*, 2006). The objective of this study was to evaluate the influence of various relative humidity and temperature conditions has on leaf spot disease severity caused by *A. helianthicola* on sunflower.

6.2.Materials and Methods

6.2.1. Pathogen cultures

Inoculations were done using the *Alternaria helianthicola* (Rao and Rajagopalan) (PPRI 11433) strain that was isolated from leaf spot infected leaves of sunflower (*Helianthus annuus* L.). The infected sunflower leaves were collected from the KwaZulu-Natal Province (S29°0456.54 E30°3601) during a field trip in 2010. A single spore *Alternaria* isolate was prepared by pouring 4 ml of sterile distilled water on a 14 days-old culture. About 2 ml of the suspension was spread uniformly on potato dextrose agar (PDA) (Merck) which was incubated at 25°C for 24 hours. The Petri dishes were examined under a Zeiss light microscope to locate single, germinated spores. The *Alternaria* cultures were maintained as conidia frozen in 20% glycerol at -20°C and as mycelial blocks in double sterile distilled water at 4°C.

6.2.2. Plant material

Sunflower (*Helianthus annuus* L.) seeds (cultivar PAN 7351) were received from Pannar, Pretoria, South Africa. The seeds were surface sterilised with 0.5% sodium hypochlorite solution for 5 min and rinsed in sterile water for one minute. Five seeds were sown in each 12 cm diameter plastic pots containing pasteurised soil (colour: light red, pH (H_2O): 6.6, pH (KCl): 6, sand: 85%, clay: 11%, silt: 4%, texture: loamy sand). The plants were maintained in the greenhouse at temperatures of 25 to 30°C and relative humidity of 50-80% with a photoperiod of 16 hours and watered daily. The plants were left to grow until 4-weeks old before use in the below experiment.

6.2.3. Evaluation of inoculation techniques

Sunflower plants were inoculated with a spore suspension of 7.2 $x10^5$ spores/ml of the *A. helianthicola* (PPRI 11433) isolate. The plants were inoculated using five various techniques:(a) leaves were sprayed with the spore suspensions until run-off; (b) the spore suspension was spread on the plants using a hockey stick; (c) mycelium growing from agar was pressed on the leaves; (d) the spore suspension was brushed onto the leaves and; (e) the leaves of the plants were pricked with a toothpick and the spore suspension was spread on the leaves using a hockey stick. The plants were covered with polyethylene bags to increase the relative humidity within

the surrounding area of the plant. The plants were maintained in the greenhouse at 25°C with a photoperiod of 16 hours. The evaluation of the symptoms was done over a period of one week. Control plants were sprayed with sterile distilled water that had no inoculum. The plants were rated according to the disease severity index (see Chapter 3, Table 3.1) developed previously.

6.2.4. The effect of temperature on radial growth of *Alternaria helianthicola*

In vitro temperature*:* The effect of temperature on the growth rate of *Alternaria helianthicola* (PPRI 11433) was investigated by taking a 5 mm^2plug from a pure culture of *A. helianthicola* grown on PDA for 7 to 14 days. The plug was placed on the centre of fresh PDA medium. Inoculated plates were incubated for 7 days at 20, 25, 30, and 40°C, respectively. Two light regimes were used, which was 8 hours dark/16 hours UV light and 12 hours UV light/dark regimes. There were eight replicates per temperature. The mycelial growth was measured daily for each plate, and then the mean colony diameter was calculated.

6.2.5. The effect of temperature on pathogenicity

In vivo temperature*:* Sunflower plants that were prepared as described previously were inoculated with a spore suspension of 7.2 $x10^5$ spores/ml of *A. helianthicola* (PPRI 11433). The conidial suspension was sprayed onto the plants until run-off with an automatic aerosol sprayer. The remaining plants were sprayed with sterile distilled water without the spore suspension until run-off as a control. The inoculated plants were placed in greenhouse compartments where the temperatures were set at 20, 25, 30, 35 and 40°C, respectively, with a photoperiod of 16 hours. The plants were initially covered with a polyethylene bag for 24 hours to maintain a high relative humidity. Disease severity was rated according to the disease severity index (see Chapter 3, Table 3.1).

6.2.6. The effect of relative humidity (RH) on pathogenicity

In vivo relative humidity*:* Sunflower plants were inoculated with a spore suspension of 7.2 $x10^5$ spores/ml of *A. helianthicola* (PPRI 11433).The conidial suspension was sprayed onto plants until run-off with an automatic aerosol sprayer. The control was prepared by spraying the plants with sterile distilled water. The inoculated plants were covered with polyethylene bagsto maintain a high level of humidity (80 to 100%) for durations of 12, 24, 48 and 72 hours. The

plants were maintained in a greenhouse at the temperature of 25°Cwith a photoperiod of 16 hours. The symptoms on the plants were evaluated at 7 days post inoculation. Diseaseseverity was rated according to the disease severity index (see Chapter 3, Table 3.1).

6.2.7. Statistical analysis

The study was done in the greenhouse and was completely randomized, with inoculation techniques, relative humidity and temperature as the treatment factors. All experiments were conducted twice in a completely randomized design with five replicates. Separation of means was done using the least significant difference (LSD) test ($P \leq 0.05$) which was computed to study the interaction effects. The disease severity was analysed using the standard analysis of variance (ANOVA), the data analysed non-parametrically by rank transformation followed by SAS's general linear model (GLM) because the rating was ordinal. The mean and variance was computed from the ranks of disease severity. The statistical analysis of this study was done for *in vivo* experiments. Severity of foliar symptoms was recorded on a 0-to-4 ordinal scale; see Chapter 3, Table 3.1 for symptom description.

6.3.Results

6.3.1. Evaluation of inoculation techniques

There were significant differences between the various inoculation techniques. Spraying the leaves with a spore suspension showed to be most effective form of artificial inoculation of *A. helianthicola* based on the median disease rating (Table 6.1). Controls of the various inoculation methods showed no infection. In a few cases, other *Alternaria* species were isolated from the infected control lesions due to the fact that seeds probably already had latent infections by these fungi. *Alternaria helianthicola* was consistently isolated from the lesions when leaves were placed on PDA.

Table 6.1: Median disease rating, mean rank, and disease severity grouping for the severity of foliar symptoms on sunflower (*Helianthus annuus* L.) caused by *Alternaria helianthicola* as influenced by various inoculation techniques.

Inoculation techniques	**Median disease rating[a]**	**Mean rank [b]**	**Disease severity grouping***
Spray	2.4	26.5	a
Brush	2.1	22.4	a, b
Agar	1.9	21.3	a, b
Prick	1.5	16.4	b
Spread	1.5	16.0	b
Control Brush	0.2	6.0	c
Control Agar	0.2	5.8	c
Control Spread	0.2	5.8	c
Control Prick	0.1	4.3	c
Control Spray	0.0	0.0	c

*Means followed by the same letter in a column indicate no significant difference. Least significant difference of median rating and mean rank was 0.6872^{a} and 7.5176^{b}, respectively. Df=21 and $P \leq 0.0001$.

6.3.2. The effect of temperature on radial growth of *Alternaria helianthicola*

The optimal growth temperature of *A. helianthicola* was 25°C. At 25°C, the growth rate of *A. helianthicola* ranged from 8.3 to 10 mm/day depending on the light regime. The cultures incubated under longer light regimes had a greater radial growth. The maximum growth temperature was 35°C (Fig. 6.1). No growth was evident at 40°C, but growth was later seen when the cultures were transferred to a 25°C incubator. At temperatures of 20, 35 and 40°C, the morphology of the *A. helianthicola* appeared to be pinkish to light brown as compared to the olivaceous green at temperatures of 25 and 30°C (Fig. 6.2). At 35°C, the growth of the pathogen seemed to be more sporadic than radial and the light regime did not influence the growth rate. The number of conidial spores obtained from each *A. helianthicola* culture washed with 5ml of distilled water was approximately 3.2×10^{7} conidia per ml at temperatures of 20 to 30°C.

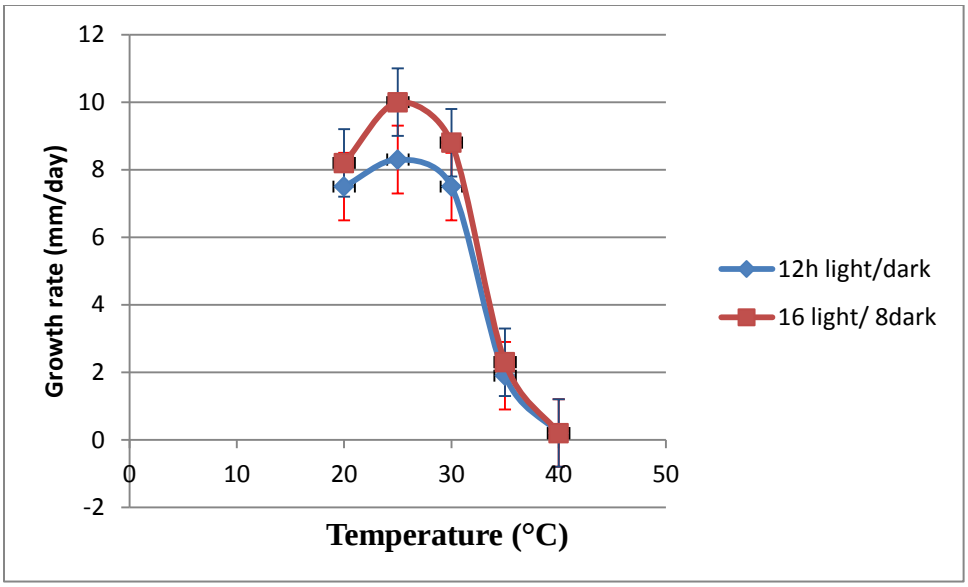

Figure 6.1: The growth rate of *Alternaria helianthicola* under various temperature conditions.

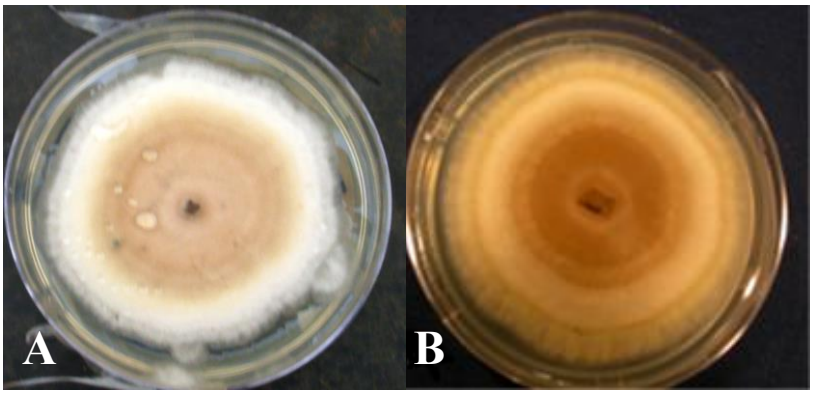

Figure 6.2: The pigmentation of *Alternaria helianthicola* at (A) 20°C and (B) 25°C.

6.3.3. The effect of temperature on pathogenicity

The optimum temperature for lesion development by *A. helianthicola* on sunflower leaves *in vivo* was 25-30°C, but the highest median disease severity was at 30°C (Table 6.2). Based on the mean rank and median disease rating, disease was seen to be least severe at 20°C. At 20°C, the symptoms were mainly seen on the inner leaf part or leaf margin (Fig. 6.3). There was a significant difference between the various temperature treatments as well as their controls ($P \leq 0.0001$).

Table 6.2: Median disease rating, mean rank, and disease severity grouping for the severity of foliar symptoms on sunflower (*Helianthus annuus* L.) caused by *Alternaria helianthicola* as influenced by temperature.

Temperature	**Median disease rating[a]**	**Mean rank [b]**	**Disease severitygrouping***
20°C	1.6	18.2	b
25°C	2.0	25.1	a, b
30°C	2.2	26.3	a
35°C	1.8	20.4	a, b
Control 20°C	0.0	10.3	c
Control 25°C	0.3	6.7	c
Control 30°C	0.0	4.5	c
Control 35°C	0.1	4.5	c

*Means followed by the same letter in a column indicate no significant difference. Least significant difference of median rating and mean rank was 0.4692^{a} and 6.5145^{b}, respectively. Df=24 and $P \leq 0.0001$.

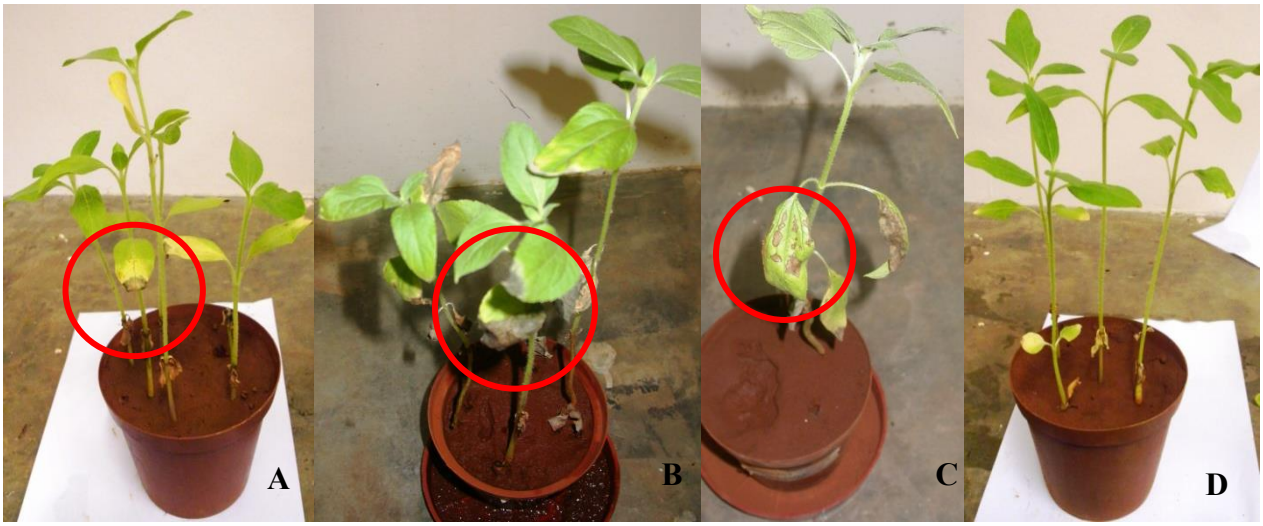

Figure 6.3: Symptoms of leaf spot disease caused by *Alternaria helianthicola* on sunflower (*Helianthus annuus* L.) at (A) 20°C; the disease was seen mainly on older leaves that began to senescence. The pathogen caused severe symptoms at (B) 25°C and (C) 30°C. No lesions were seen on the control plants (D).

6.3.4. The effect of relative humidity (RH) on pathogenicity

Disease severity increased with increasing length of relative humidity period at 25°C, but there was no significant difference between 12, 24, 48 and 72 hour durations (Table 6.3). Alternaria leaf spot disease was seen to be most severe for the plants covered with polyethylene bags for 48 hours and the least for 12 hours. There was a significant difference between the treatment and their control ($P \leq 0.0001$).

Table 6.3: Median disease rating, mean rank, and disease severity grouping for the severity of foliar symptoms on sunflower (*Helianthus annuus* L.) caused by *Alternaria helianthicola* as influenced by relative humidity.

Relative humidity	**Median disease rating [a]**	**Mean rank [b]**	**Disease severity grouping***
12 hrs	1.8125	12.625	a
24 hrs	2.0625	18.750	a
48 hrs	2.1250	18.750	a
72 hrs	1.9375	15.875	a
Control 12 hrs	0.0000	3.500	b
Control 24 hrs	0.0000	3.500	b
Control 48 hrs	0.1250	5.500	b
Control 72 hrs	0.1250	5.500	b

*Means followed by the same letter in a column indicate no significant difference. Least significant difference of median rating and mean rank was 0.3251^{a} and 6.5131^{b}, respectively. Df=16 and $P \leq 0.0001$.

6.4. Discussion

This study focused on determining the environmental conditions that are suitable for *A. helianthicola* to cause leaf spot disease sunflower, as varying *Alternaria* species differ in terms of growth rate, timing of sporulation, and the number of spores produced, and optimal growth conditions for spore germination and growth (Pryor *et al.*, 2003).The results of this work indicate that *Alternaria helianthicola* has the ability of causing severe leaf spot symptoms on sunflower provided the environmental conditions are suitable for instigating disease.

The spraying until run-off technique was used for subsequent experiments as it had the highest mean in terms of disease severity, although application could cause conidia to run-off the leaves due to excess application volume, resulting in reduced conidial deposition (Green and Bailey, 2000). The advantage of the spray until run-off technique is it is less abrasive as compared to other inoculation techniques such as pricking and brushing that cause mechanical damage to the leaves. Spraying is more ideal than other inoculation techniques because it simulates how *Alternaria* species are disseminated in the field, through rain splashes (Agrios, 2005).

The radial growth *A. helianthicola* was 10mm/day at 25°C and this did not concur with the radial growth of typical small-spored *Alternaria* species which is about 5mm/day under standard conditions (cultured on PCA and incubated at 22°C) (Simmons, 1995). The lighting regimes and the type of medium agar used may have played a role in the growth rate differences between these respective studies. This was evident because the *A. helianthicola* cultures that were incubated at 25°C with more light duration had more mycelial growth of 10mm/day and 8.3 mm/day, if the plates were incubated at 16 hours and 12 hours light, respectively. The radial growth rate was not affected by light at 20 and 35°C. This is because light enhances the formation of conidiophores and vegetative growth of the hyphae, but it inhibits conidial formation. In the second phase, the conidia are formed in darkness (Rotem, 1994; Pryor and Michailides, 2001). Light inhibits sporulation when the temperatures are relatively high, but not when temperaturesare lower (Masangkay *et al.*, 2000).

Alternaria helianthicola cultures produced a pinkish pigmentation at temperatures of 20°C and 35°C (Fig. 6.6). This may be due to the fact that most *Alternaria* species exhibit considerable morphological plasticity that is dependent on cultural conditions of substrate, temperature, light and humidity. The optimal temperature for disease development was 30°C *in vivo*. Based on the *in vitro* temperature tests, 25°C was expected to be the optimal temperature because *A. helianthicola* had a highest radial growth rate in that condition. There was no significant difference in disease severity between the incubation at 25 and 30°C, this implies that growth rate of the *A. helianthicola* may have no effect on the infection of the sunflower leaves. This is because the optimal temperatures for germ tube formation, appressorial formation, and for hyphal growth during infection are different (Everts and Lacy, 1996; Blodgett and Swart, 2002) and infection is dependent upon germ tube formation and appressorial formation morethan hyphal growth.

The effect of the duration of relative humidity on the leaf spot disease was evaluated by placing polyethylene bags on inoculated plants over intervals of 12, 24, 48 and 72 hours. Symptoms were seen on plants from 12 hours post inoculation. Usually 10-12 hours of wetting is needed for substantial infection (Timmer*et al.,* 2003), although other species require long moisture periods of 48-72 hours. Free moisture facilitates epidemics under all temperatures, but the higher the temperature, the quicker the spread of the epidemics (Rotem, 1994; Reis *et al.*, 2006). There was no significant difference between 24 and 72 hours of relative humidity (Table 6.3). This implies that after a certain duration of high relative humidity, relative humidity will cease to play a role in disease severity provided infection has already been initiated. Studies done on Canada thistle (*Cirsium arvense* L.) demonstrated that free water was optimal, but not essential for the infection of Canada thistle by *A. cirsinoxia* (Green and Bailey, 2000).

In conclusion, when doing *in vivo* greenhouse trials, the type of inoculation technique should be taken into consideration and this technique should mimic the natural infection process. The optimum temperatures for *Alternaria* to infect sunflower range from 25 to 30°C. Approximately 12 hours of high relative humidity is required for initiation of the infection process, provided the temperature factor is optimal since as the temperatures decline, longer periods of wetting are needed for disease to occur (Timmer*et al.*, 2003).

6.5. References

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Chapter 7: General discussion

Sunflower (*Helianthus annuus* L.) is the third most important crop in South Africa after maize (*Zea mays* L.) and wheat (*Triticum aestivum* L.) but is the most important oilseed-crop after soybean and rapeseed (Grains South Africa, 2010). Sunflower is preferred over soybean and rapeseed because of its poly-unsaturated oil which minimizes the chances of cholesterol. Sunflower has been shown to grow in a wide range of environmental conditions (Weis, 1983; Robellen *et al.*, 1982). Sunflower is grown mainly in five areas in South Africa, which include the Limpopo, North West, Mpumalanga, Gauteng and the Free State provinces (DAFF, 2012).

During the summer rainfall times, a survey in sunflower farms in Greytown (February 2010) and Potchefstroom (April 2011) showed the prominence of a severe leaf spot disease on sunflower. The symptoms resembled those caused by *A. helianthi* (Hanford) Tubaki and Nishihara, which were characterized by irregular dark, necrotic lesions with a grey centre that is surrounded by a chlorotic halo on the leaves, stem, petioles and capitulum. Severely infected lesions become larger by coalescing, leading to blight and defoliation and death of plants (Mirza *et al.*, 1984).The causal agent of the disease was identified as *A. helianthicola*. *A. helianthicola* was first described in India by Rao and Rajagopalan (1977) and later in Taiwan (Wu and Wu, 2003) and Croatia (Vrandecic*et al.*, 2011).

The Infectoria species-group forms part of the fourteen small-spored *Alternaria* species-groups which were characterized and arranged by Simmons (1990; 1993 (Roberts); 1994; 1995) according to their three dimensional sporulation patterns, size and other cultural characteristics. The most common *Alternaria* species associated with sunflower leaf blight is *A. helianthi* (Prasad *et al.*, 2008). Detailed infection aspects of the small-spored *Alternaria*on sunflower and their pathological effects on foliar disease severity and seed germination have been little studied in South Africa.

Hence, the objectives of this study was to determine the causal agent of the leaf spot disease on sunflower and compile a disease severity index of its symptoms (Chapter 3), to determine the

percentage germination of thirteen sunflower seed lots collected from different farms in the various provinces of South Africa and to determine whether seed infection by *Alternaria* spp. had an effect on germination on those seed lots (Chapter 4), to identify *Alternaria* isolates recovered from sunflower leaves and seeds obtained from various sunflower growing areas of South Africa by molecular identification techniques (Chapter 5) and to evaluate the influence of various environmental conditions on leaf spot disease severity caused by *A. helianthicola* on sunflower (Chapter 6).

Artificial inoculation of the sunflower plant in the greenhouse produced similar leaf spot disease symptoms to those originally isolated from KwaZulu-Natal, and re-isolation from the inoculated leaves proved that *A. helianthicola* was the causal agent of leaf spot and blight of sunflower. Koch's postulates were thus completed. The results from Chapter 3 of this study showed that *A. helianthicola* is capable of severe leaf spot symptoms on sunflower and can be as damaging as *A. helianthi.*

Seed quality tests ensure that the farmer uses the best quality seeds to avoid spreading disease, as many plant pathogens are disseminated predominantly by seed (Neergaard, 1977). The environmental conditions of the areas where the sunflower seed lots used in this study were harvested from, had a mean temperature of approximately 30°C and a mean relative humidity above 85% (Agrometeorology Staff, 2012). *Alternaria* species are prevalent in these warm and wet conditions (Reis *et al.*, 2006). Germination percentages ranged from 60 to 94% and the agar plate method showed that seed infection by *Alternaria* spp. infection ranged from 18 to 98% caused by various small-spored *Alternaria* spp. PAN 7050 (Greytown) and PAN 7049 were the least infected seed lots, and had the highest germination potential. The correlation coefficient between seed germination and seed infection was -0.56. The correlation implies seed infection may cause the decrease in germination. However, 56% cannot be regarded as a strong correlation, which is evident since some seed lots such as NK ferti and Agsun s661 had both a high percentage infection and a high percentage germination.The factors that may have played a role in the weak correlation are that *Alternaria* species are saprophytic (Peever*et al.,* 2004) and some *Alternaria* species may cause quiescent infection until the environmental conditions are

conducive for disease (Rotem, 1994), and while other *Alternaria* species are only mere contaminants of the seed coat but do not cause disease (Neergaard, 1977).

The high incidence of *Alternaria* infestation on the sunflower seeds may be favoured by the warm and wet summer field conditions. During this period the environmental conditions favour the proliferation of *Alternaria* species (Reis *et al.*, 2006). Over the past five years during the sunflower growing season in Ventersdorp, Viljoenskroon,Greytown and Pretoria the average temperature and the average relative humidity has been 30°C and 92%, respectively (Agrometeorology Staff, 2012). Whereas Bloemfontein had an average temperature and relative humidity of 25°C and 80% (Agrometeorology Staff, 2012), and seed infection by *Alternaria* species on sunflower seed lots grown in Bloemfontein was less than 70%. The small-spored *Alternaria* species that were isolated from the seeds included *A. helianthicola, A. alternata* and *A. tenuissima.*

Seed component plating tests showed that the *Alternaria* species were more prevalent in the embryo and cotyledon than on the seed coat. High levels of internal infection indicate that the fungus was not a chance contaminant but its presence resulted in the direct attack of the seeds. The seed may have been infected systemically from the mother plant. During systemic infections, the fungus grows into the ovule and later develops from the seed further into the seedling to cause seedling blight (Neergaard, 1977). However, Szopinska*et al.*, (2007) stated that the seed coat is a common site of infection by most seed-transmitted Mitosporic fungi (formerly known as Fungi Imperfecti). The seed coats may have been infected from the outside by conidia being disseminated by wind to young florets of healthy heads. In some cases the pathogen may be found contaminating the seed surface (Suryanarayana, 1978). The lower *Alternaria* seed coat infection percentage in this study may have been caused by the sodium hypochlorite surface disinfection technique, as reported by Szopinska and Bralewski (2006).

The sexual stage of most *Alternaria* spp. is unknown. Generally *Alternaria* species exist in the vegetative phase and reproduce asexually. This is why they display a low phenotypic variation (Guo *et al.*, 2004).Identification of many of the small-spored *Alternaria* species still offers considerable difficulties owing to their similarities and polymorphism even in pure cultures

(Simmons, 1992) and these species produce small spores aggregating in branching chains with morphological characteristics that overlap. Thus, the small-spored *Alternaria*species are often misidentified (Pryor and Michailides, 2001). Pathogen and disease diagnosis are fundamental to virtually all suspects that relate to plant pathology (Ma and Michailides, 2004). Morphological and molecular techniques were used in this study to characterize small-spored *Alternaria* species that were isolated from leaf spot infected leaves and sunflower seeds.

The *Alternaria* isolates that were under study were placed into three groups. These groups were *A. helianthicola*, *A. tenuissima* and *A. alternata*. The conidial sizes of these *Alternaria* spp. ranged from 40-70 µm in length and 12-20 µm in width and they all formed chains. Cultures of *A. helianthicola* sporulated abundantly as bushy clumps of geniculate and branching secondary conidiophores produced by and separated by secondary conidia. *Alternaria tenuissima* conidia had short apical conidiophores that arose singly from the mycelium. *Alternaria alternata* had medium size conidiophores that branched out, with each conidiophore carrying a chain of conidia. The colony growth pattern of these three *Alternaria* groups was circular with concentric circles within the colonies due to the alternating 12 hours light and darkness regimes. However, culture pigmentation was not taken into consideration for morphological identification because isolates of *Alternaria* are genetically variable and any given mycelium may become heterokaryotic (Slavov*et al.*, 2004).

To confirm the identity of the *Alternaria* isolates, additional identification methods using molecular techniques were employed. Five *Alternaria* isolates were identified by using three gene regions of ITS rDNA operon, β-tubulin and EF-1 genes. The ITS region showed high similarity among the *Alternaria* species such as *A. alternata*, *A. brassicae*, *A. mali*, *A. gaisen, A. citri, A. lini, A. brassicicola, A. tenuissima, A. arborescens* and *A. longipes* supported by a high bootstrap value of 100%. These results concur with those of Pryor and Michailides (2001), who reported that the ITS region failed to resolve small-spored *Alternaria* species as phylogenetically distinct from *A. alternata* due to the minimal variation in their nucleotides. In *Alternaria* species taxonomy, the ITS region is recommended only for preliminary identification as it cannot resolve or segregate small-spored *Alternaria* species. The β-tubulin gene and the EF-1α gene were able to resolve or segregate small-spored *Alternaria* species. The pathogens that were isolated from

the infected leaves and seeds were identified as *A. alternata*. There were no available sequences for *A. helianthicola* from the NCBI GenBank, thus no comparisons could be made for this species.

Inoculation techniques were evaluated in the study to obtain an effective technique that simulates the natural infection of *Alternaria* species in the field. The spray until run-off technique is recommended for the application of *Alternaria* species for foliar inoculation as it is less abrasive as compared to other inoculation techniques such as pricking and brushing that cause mechanical damage to the leaves. Spraying also simulates how *Alternaria* species are disseminated in the field, through rain splashes (Agrios, 2005).

Different *Alternaria* species have life histories, such as growth rate, timing of sporulation, and the number of spores produced, and optimal conditions for spore germination and growth (Pryor *et al.*, 2003). The *in vitro* radial growth rate of *A. helianthicola* was 10mm/day and 8.3 mm/dayat 25°C when incubated under 16 hours and 12 hours light, respectively. But the radial growth rate was not affected by light at 20 and 35°C. This is because light enhances the formation of conidiophores and vegetative growth of the hyphae, but it inhibits conidial formation (Rotem, 1994; Pryor and Michailides, 2001). However, light inhibits sporulation when the temperatures are high but not when they are low (Masangkay *et al.*, 2000).

Alternaria species thrive in areas of high relative humidity and temperature. There was no significant difference in leaf infection among relative humidity durations of 24, 48 and 72 hours. Green and Bailey (2000) demonstrated that free water was optimal, but not essential for the infection *Alternaria* species. The optimal temperature for causing disease severity was 30°C *in vivo*. This is because the optimal temperature for germ tube formation and appressorial formation vary to those of hyphal growth during infection (Everts and Lacy, 1996) and infection is mainly dependent upon germ tube formation and appressorial formation more than hyphal growth.

In conclusion, the incidence of *Alternaria* species shows that these fungi are common in all sunflower producing areas of South Africa and are not confined to a particular cultivar of sunflower as they were detected in all the seed lots used in this study. The presence of the fungi

in the embryo and cotyledon of the seeds suggests that fungicidal control may be achieved provided chemicals with penetration abilities or soak treatments that can eliminate internal infections. However, most importantly the use of pathogen-free seeds will be an essential component of an integrated management of seed-borne *Alternaria* species on sunflower. Future research should include the use of chemical segregation of *Alternaria* species, the study of the infection process of these isolates both on the seeds and leaves, fungicide treatments, possible biocontrol agents, development of genetic resistance and other control measures.

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